TYPE 1 ANGIOTENSIN II RECEPTORS IN BOVINE ADRENOCORTICAL CELLS.

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A thesis submitted in partial fulfilment
of the requirement for the degree of
Doctor of Philosophy

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DECLARATION OF ORIGINALITY

I declare that the composition of this thesis and the work presented herein is my own.

Ghislaine Clare Dell.

ACKNOWLEDGEMENTS

I would firstly like to thank my supervisors, especially Dr. B.C. Williams and Dr. S.W. Walker, for all their patient help, guidance and enthusiasm throughout this project. Grateful thanks are also due to Dr. S.D. Morley, whose molecular biology expertise helped greatly.

The many colleagues and friends who have given me both help and moral support I am extremely grateful to. Without the assistance of the meat inspectors at Gorgie abattoir the project could not have succeeded and I would like to thank them. I have worked in three different departments and naming all those involved would take up a lot of room. However, I would particularly like to mention Moira, Forbes, Vanessa, Elaine and Stuart at Clinical Biochemistry, RIE; Nicola at Medicine, WGH, and Alison, Allan, Julia and Davy at the CGR; also not forgetting Matt whose patience with my lack of familiarity with computers was boundless! I would also like to acknowledge Dr. Margaret McIntyre for histopathological analysis of bovine adrenal gland slices.

I should also like to acknowledge and thank Dr. A.F. Smith (Clinical Biochemistry, RIE), Professor A. Ferguson, (Medicine, WGH) and Dr. J.J. Mullins (CGR) for allowing me to undertake this project and for use of laboratory facilities within their departments. I am also grateful to the Medical Research Council for financial support in the form of a 3-year stipend.

Finally, I would also like to thank my family and friends for encouragement throughout the project - the support from these and everyone mentioned was invaluable.

Abbreviations

12-HETE	12-hydroxyeicosatetraenoic acid
8Br-cAMP	8-bromo-cyclic AMP
A+P	A23187 + PMA
A23187	calcium ionophore
A260	absorbance at 260nm
AC	adenylate cyclase
ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
AI	angiotensin I
AII	angiotensin II
AIII	angiotensin III
AIV	angiotensin IV (angiotensin II (3-8))
AT_1	type 1 AII receptor
AT_{1a}	type 1a AII receptor
AT_{1b}	type 1b AII receptor
AT_{1c}	type 1c AII receptor
AT_2	type 2 AII receptor
AT_3	type 3 AII receptor
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
B_{max}	maximum binding constant (number of receptors)
bp	base pairs
BSA	bovine serum albumin
C.I.	confidence interval
CAM-kinase II	calcium-calmodulin kinase II
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cpm	counts per minute
CPSR-1	controlled process serum replacement-1
DAG	diacylglycerol
ddA/T/C/G/ITP	dideoxy A/T/C/G/ITP
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
dNTP mix	deoxynecleoside mix (dA,C,G,TTP)
DOC	deoxycorticosterone
dpm	disintegrations per minute
dr	dose ratio
DTT	dithiothreitol
EBS	Earle's balanced salt solution
ECF	extracellular fluid

EDTA	ethylene diamine tetra-acetic acid
EGF	epithelial growth factor
EtBr	ethidium bromide
FCS	foetal calf serum
FSH	follicle-stimulating hormone
G3PDH/GAPDH	glyceraldehyde triphosphate dehydrogenase
GTP	guanosine triphosphate
IC_{50}	inhibition constant (for 50% response)
IGF-1	insulin-like growth factor-1
IP_3	inositol 1,4,5-trisphosphate
Jak-STAT	'jurkat-activated kinase' - Signal Transducers and Activators of Transcription
K_B	antagonist dissociation constant
kb	kilobase pairs
K_D	dissociation constant
KKL	Klenow Kinase Ligase
LH	luteinising hormone
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
mRNA	messenger RNA
NO	nitric oxide
PA	phosphatidic acid
pA_2	negative logarithm of [antagonist] producing dr shift of 2
$pAT_1.n$	plasmid containing AT_1 insert
PBS	phosphate-buffered saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phosphatidyl ethanolamine
PEG	polyethylene glycol
PG	prostaglandin
PI	phosphatidyl inositol
PIP	phosphatidyl inositol 4-phosphate
PIP_2	phosphatidyl inositol 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
RAS	renin-angiotensin system
RIA	radioimmunoassay
rt-PCR	reverse transcriptase-PCR
s.e.m.	standard error of the mean
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
SSC	saline sodium citrate

SSPE	saline-sodium phosphate-EDTA buffer
TAE	Tris-acetate-EDTA butter
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
TNE	Tris-NaCl-EDTA buffer
vsm	vascular smooth muscle
vsmc	vascular smooth muscle cells
zf	zona fasciculata
zfr	zona fasciculata/reticularis
zg	zona glomerulosa
zr	zona reticularis

Abstract

Angiotensin II, the main effector peptide of the renin-angiotensin system, is an important hormonal regulator of blood pressure and electrolyte balance. It acts through receptors in its many target organs to exert a wide range of effects. Foremost amongst these are stimulation of the zona glomerulosa (zg) of the adrenal gland to secrete the mineralocorticoid aldosterone, and contraction of vascular smooth muscle, particularly resistance arterioles. The physiological significance of AII receptors in the zonae fasciculata/reticularis (zfr) of the adrenal cortex is less well characterised, despite the fact that AII can directly stimulate glucocorticoid secretion in a number of species. The recent use of non-peptide AII antagonists and molecular cloning have established that AII receptors can be divided into two classes : AT_1, which mediate the cardiovascular actions of AII, and AT_2, for which no clear physiological function has been found.

It has been argued that the bovine zg and zfr are the most appropriate cellular models for human adrenocortical cells. This thesis presents modifications to improve the established methods for isolation and culture of zg cells, and characterises responses to steroidogenic agonists during short-term primary culture. The modified zg culture was used as a tool to pharmacologically characterise the type 1 AII receptor using Schild analysis in order to obtain pA_2 values for a selective AT_1 receptor antagonist, losartan (DuP753). The pA_2 value is a measure of antagonist affinity which should be constant for the antagonist at a particular receptor subtype. The pA_2 values obtained were compared to both pA_2 values previously reported for bovine zfr cell AT_1 receptors, and pA_2 values calculated from cultured rat mesenteric artery smooth muscle cells. No significant difference was found in the pA_2 values (zg : 7.02 +/- 0.20; vsmc : 7.28 +/- 0.23), indicating that pharmacologically the receptors are indistinguishable.

The bovine AT_1 receptor was then cloned, from bovine spleen, using the published bovine adrenal AT_1 receptor sequence, by PCR. The resulting clones were sequenced and confirmed as AT_1 receptors. The DNA was then used to probe for AT_1 receptor mRNA expression in primary cultures of bovine zfr cells using Northern blot analysis. Specific agonists for the adrenal cortex, and second messenger analogues, were employed in these experiments.

AII caused homologous downregulation (86%) of its receptor mRNA, an action found to be mimicked by stimulation of protein kinase C and calcium influx (91%). A cyclic AMP analogue (8-bromo-cyclic AMP) also caused downregulation of AT_1 receptor mRNA. In contrast, AT_1 receptor mRNA was upregulated by incubation of the cells with insulin-like growth factor-1 (IGF-1) and potassium. Finally, the possibility that adrenal corticosteroids could exert a short-loop feedback effect on AT_1 receptor mRNA was analysed. Cortisol, but not aldosterone, significantly downregulated (41%) the level of AT_1 receptor mRNA, which indicated a possible zone-specific effect on the regulation of AT_1 receptors in the adrenal cortex.

This thesis therefore presents data on the nature and regulation of type 1 angiotensin II receptors in bovine adrenocortical cells. These studies suggest that the physiological regulation of AII receptors within the adrenal cortex is more complex than has been previously considered.

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Chapter 1 : Introduction

1.1 Angiotensin II

1.1.1 Discovery and history

The octapeptide now known as angiotensin II was originally discovered in 1939, by Braun-Menendez and by Page and Helmer (Peach, 1977), who gave it the names hypertensin and angiotonin to indicate that this humorally-active substance acted to increase blood pressure by constricting arteries. By 1958, it had been given the compromise nomenclature of angiotensin, later angiotensin II as the manner of its formation in a cascade was elucidated. Between 1954 and 1957, its peptide structure was reported :

N-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-C

and synthesised by Skeggs, Peart and Bumpus (Peach, 1977); the C-terminal phenylalanine was found to be crucial for biological activity. Since then, much has become known about its formation, regulation, functions and mechanism of action; these topics form the basis of parts 1.1 to 1.4 of this introduction.

1.1.2 Formation and regulation : the renin-angiotensin system

The formation of angiotensin II is controlled and regulated by the renin-angiotensin system (RAS). This humoral system is involved in the control of vascular tone, volume homeostasis and electrolyte balance (Peach, 1977; Vallotton, 1987; Ferrario, 1990; Lifton, 1996). The RAS was originally thought to be purely systemic, but evidence has now been demonstrated of local RAS in other organs including the adrenal gland, heart, kidney, brain and gonads (Vinson *et al.* 1995b; Griendling *et al.* 1993b).

BIOCHEMISTRY OF THE RENIN-ANGIOTENSIN SYSTEM

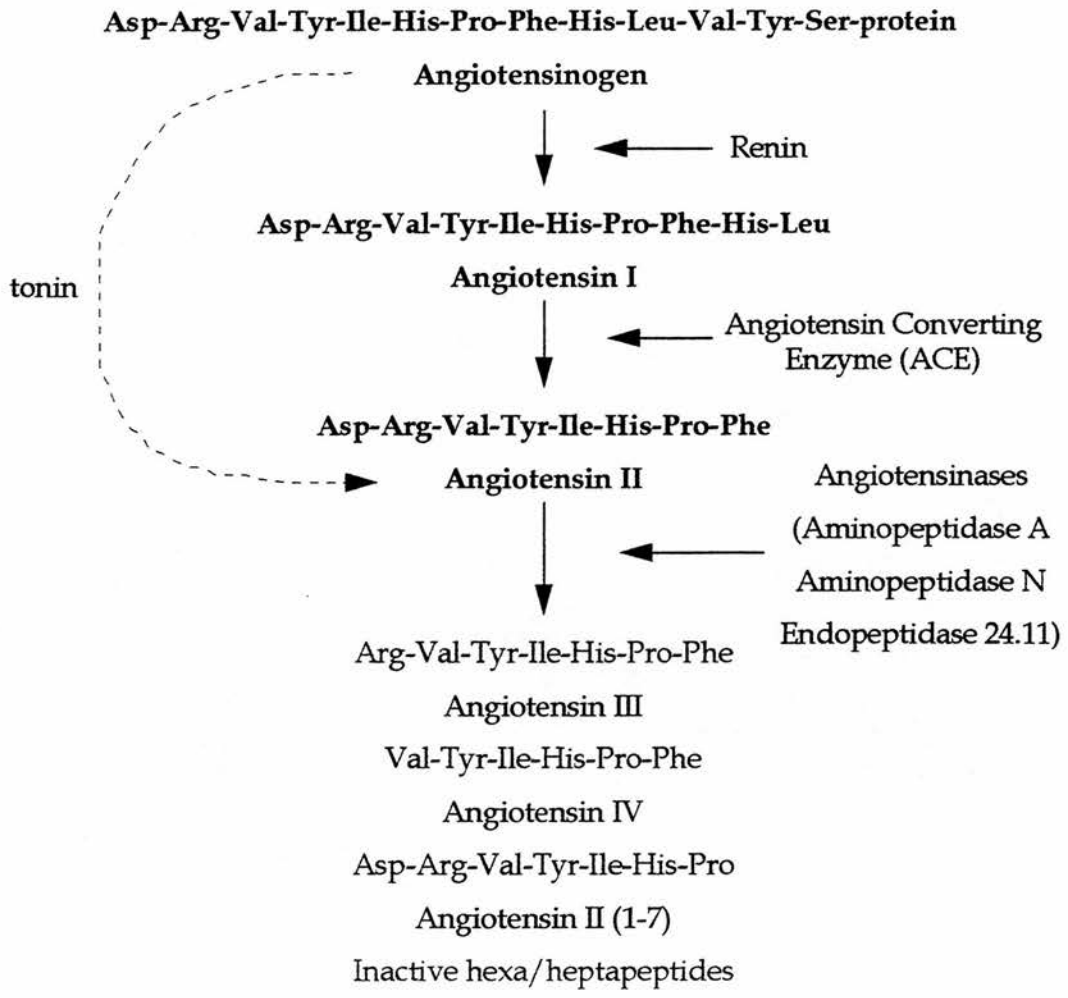


Figure 1.1a

The biochemistry of the renin-angiotensin system, showing the enzymes involved and the peptides they generate.

PHYSIOLOGY OF THE RENIN-ANGIOTENSIN SYSTEM

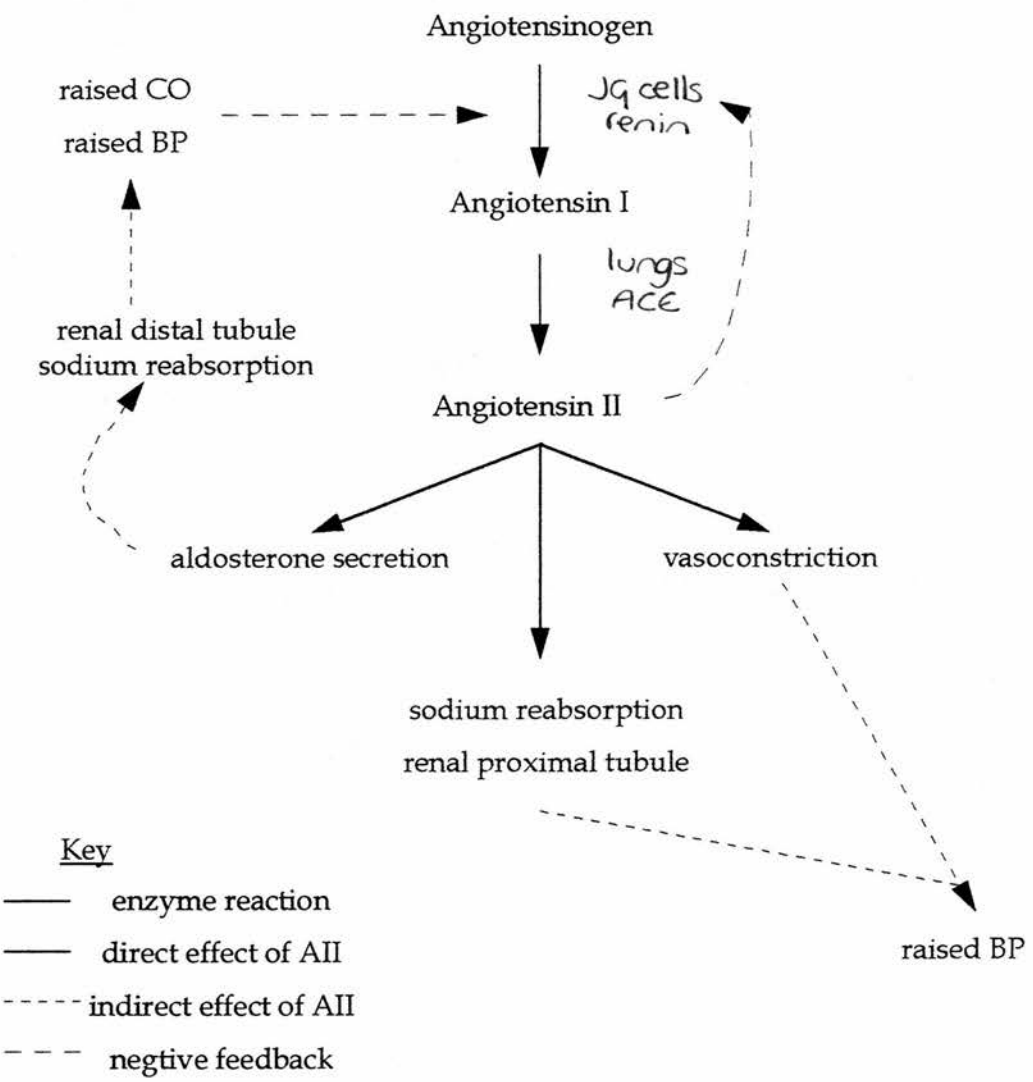


Figure 1.1b

The physiology of the renin-angiotensin system : its main effects with regard to the cardiovascular system. (JG cells = juxtaglomerular cells of the kidney)

1.1.2.1 The systemic RAS

The components of the RAS are shown in figure 1.1. Renin, an aspartyl protease (MW 38kDa), is secreted by the juxtaglomerular cells of the kidney (Peach, 1977), and cleaves angiotensinogen to produce angiotensin I (AI) by hydrolysing the Leu^{10}-Val^{11} bond. Renin is stored in granules and secreted into the plasma either as active renin or inactive prorenin, which is converted to active renin, possibly by kallikrein.

Renin release is stimulated by a baroreceptor reflex, low [Na^+] via the macula densa, the sympathetic nervous system, high plasma potassium or lowered arterial pressure in the kidney. A negative feedback loop also operates in the RAS : raised AII levels inhibit further renin release, as well as a positive loop, possibly mediated by the activation of the angiotensinogen gene by raised AII levels (Li & Brasier, 1996).

Angiotensinogen is the only substrate for renin and is a glycoprotein of the α2-globulin class, belonging to the serine protease inhibitor family (serpins) (Griendling *et al.* 1993b). In humans, it is mainly found in the liver and has a MW of 55-65kDa depending on the degree of glycosylation. Its plasma concentration is close to the Michaelis-Menten constant for renin, and its concentration therefore critically determines the amount of AI generated (Peters, 1995).

The final part of the cascade produces the biologically active octapeptide, angiotensin II, through the action of angiotensin converting enzyme (ACE) on AI. ACE is a zinc metallopeptidase (MW60kDa) which cleaves the N-terminal dipeptide fragment from AI. It is found predominantly in lung endothelial cells, and requires chloride ions for efficient catalysis. ACE is identical to kininase II, which inactivates bradykinin (Griendling *et al.* 1993b). AII can also be formed directly from angiotensinogen, by the action of the enzyme tonin. (Ferrario, 1990)

AII, the main effector peptide of the RAS, exerts multiple potent effects, mainly on the cardiovascular system. It has a short half-life in plasma (2-3 min) and is quickly degraded by a combination of endopeptidases and aminopeptidases to shorter fragments. Aminopeptidase A acts upon AII to yield AIII (des-Asp^1-AII), which is also physiologically active (4-5 times less potent in vasoconstriction, equipotent to AII in facilitation of sympathetic transmission but more potent than AII in stimulation of aldosterone release : (Peach, 1977)). AIII has a shorter half-life than its parent AII and is degraded to AIV (Angiotensin 3-8) by non-selective aminopeptidase N. AIV has also been reported to have some central effects (Harding *et al.* 1992; Harding *et al.* 1994). Angiotensin (1-7), produced by the action of endopeptidase 24.11 on AII, has cardiovascular effects, in part antagonistic to those of AII (Benter *et al.* 1993). Further degradation of these fragments does not yield any other active components of the system.

AII exerts its effects by interacting with specific receptors on the cell membrane of its target tissues. These receptors stimulate the calcium/inositol trisphosphate second messenger system to generate the multitude of effects elicited by AII, including vasoconstriction and aldosterone secretion, and are therefore the final components of the RAS. AIII, which is also physiologically active, binds to the same receptors as AII, but with different affinity.

1.1.2.2 Local RAS

Local, or tissue, RAS function essentially similarly to the systemic RAS described above. The local generation of AII by tissue RAS could allow individual functions of AII to be performed without exerting inappropriate systemic responses in other tissues (Vinson *et al.* 1995b). These tissue RAS could also serve as a means of 'fine tuning' the actions of AII to a specific organ or physiological event (Griendling *et al.* 1993b), contributing significantly to para-and autocrine mechanisms in the regulation of the

cardiovascular and steroidogenic systems (Paul, 1994; Chiou *et al.* 1995). The evidence for the existence of these local RAS is strong in that all the components of the system have been shown to be present, and, in some cases, synthesised, in the tissues in question. However, renal renin is avidly taken up by many tissues, especially the vasculature (Griendling *et al.* 1993b) and therefore, although there may be a functional local RAS in the vasculature, it may not be a true RAS (in the sense that all the components have been synthesised in the tissue), unlike that demonstrated in the brain (Vinson *et al.* 1995b).

In some tissue RAS, for example the cardiac and vascular RAS, local effects would be difficult to separate from systemic effects. Where the two are compartmentalised (for example in the brain) it is easier to see how the local system could regulate effects (in this example, drinking behaviour) over a longer period of time in comparison with the systemic system. Thus, these local RAS could be of significance in responding to physiological demand independently of the systemic RAS.

1.1.3 Functions of AII

The principal functions of AII are vasoconstriction and stimulation of aldosterone secretion. It has many additional effects, primarily on the cardiovascular system although independent of vasoconstriction and aldosterone. These actions have been demonstrated *in vivo* and represent a profound effect of AII on blood pressure and volume regulation. Other more recently discovered actions, e.g. effects on sperm motility, bear no relation to these cardiovascular effects.

1.1.3.1 Vasoconstriction

Angiotensin II induces an increase in both systolic and diastolic blood pressure by constricting vascular smooth muscle, particularly in resistance

vessels and small arterioles. The precapillary arterioles of the skin, splanchnic region and kidney are preferentially constricted, with a resultant increase in peripheral resistance (Peters, 1995). The conduit arteries, however, also possess receptors for AII and will constrict in response to AII *in vitro* (Dzau & Safar, 1988). AII is 40 times more potent as a vasoconstrictor than noradrenaline and before the discovery of endothelin was the most potent pressor substance known. AII also raises blood pressure and constricts arteries indirectly, through a centrally-mediated mechanism by which it facilitates noradrenergic neurotransmission, involving the area postrema (Scroop *et al.* 1971).

1.1.3.2 Steroid secretion

AII stimulates secretion of the mineralocorticoid aldosterone from the zona glomerulosa of the adrenal gland (Laragh *et al.* 1960), and further stimulates the synthesis of aldosterone by increasing the conversion of cholesterol to pregnenolone (Aguilera & Marusic, 1971), as well as later stages in the pathway, for example the 18-hydroxylation of corticosterone (Capponi *et al.* 1994; Lebrethon *et al.* 1994). The aldosterone produced stimulates the kidney to retain sodium, acting in the distal convoluted tubule, where it induces loss of potassium in exchange for sodium. In addition to its well characterised effect on mineralocorticoid secretion and synthesis, AII is also capable of stimulating glucocorticoid secretion in some species, both *in vivo* and *in vitro* (McKenna *et al.* 1978; Mason *et al.* 1979; Williams *et al.* 1989; Bird *et al.* 1992; Naville *et al.* 1993; Lebrethon *et al.* 1994), acting on specific AII receptors in the zona fasciculata/reticularis of the adrenal cortex.

1.1.3.3 Independent cardiovascular effects

AII exerts a positive inotropic effect on the heart (originally described by Fowler & Holmes, 1964) by opening voltage-sensitive calcium channels. It further acts to raise blood volume by stimulating drinking behaviour, acting on the subfornical organ of the brain (Peach, 1977), and stimulates the posterior pituitary to secrete vasopressin (Malvin, 1971). A direct effect of AII on the kidney has also been demonstrated, showing an increase in sodium reabsorption and stimulation of Na^+/H^+ exchange at proximal tubules (Peters, 1995). AII has a differential effect on blood flow in different vascular regions : it decreases mesenteric and cerebral blood flow while having a variable effect on blood flow in the renal cortex (Peach, 1977; Vallotton, 1987; Ferrario, 1990). It also stimulates catecholamine release from the adrenal medulla (Peach, 1977).

1.1.3.4 Growth effects

Angiotensin II has long been known to have hypertrophic and hyperplastic effects. It has been shown to mediate vascular smooth muscle growth (Bunkenburg *et al.* 1992), leading to speculation that AII plays a significant role in cardiovascular hypertrophy and remodelling. Evidence that AII is also involved in fibroblast proliferation has lent further weight to this (Brilla *et al.* 1994; Schorb *et al.* 1993; Hsueh & Do, 1994), as has the demonstration that AII induces cardiac myocyte necrosis (Tan *et al.* 1991). The hypertrophic and, in some cases, mitogenic influence of AII is not restricted to the cardiovascular system. Similar effects have also been reported in bovine adrenocortical cells (Linas *et al.* 1990; Clyne *et al.* 1993), rat kidney tubular and mesangial cells (Hsueh & Do, 1994; Wolf, 1994). AII also mediates stretch-induced cardiac hypertrophy (Peters, 1995), in addition to stimulating immediate-early genes such as *c-fos* and *c-jun* (Peters, 1995; Lyall *et al.* 1992) and growth factor transcription and production (Wolf,

1994). However, AII has also recently been shown to play a part in apoptosis (Yamada *et al.* 1996).

1.1.3.5 Non-cardiovascular effects

These actions, seemingly unrelated to the main role of AII, may be more connected with the local RAS described in section 1.1.2. Angiotensin II has been shown to impair retention of shock-avoidance responses and to inhibit hippocampal long-term potentiation (Lee *et al.* 1995; Wayner *et al.* 1995). It will also stimulate production of progesterone in ovarian granulosa cells (Hsueh & Do, 1994) and has been postulated to play a part in fertility after the observation that AII antagonists inhibit ovulation (Peterson *et al.* 1993). The recent discovery of AII receptors in human sperm that display increased motility after stimulation by AII also leads to the conclusion that AII has an important role in this process (Vinson *et al.* 1995b). Angiotensin II also contracts non-vascular smooth muscle, and there is a high concentration of AII receptors in the uterine musculature (Aguilera *et al.* 1980); it is also known to increase gluconeogenesis (Vallotton, 1987) in addition to facilitating sympathetic neurotransmission (Vallotton, 1987).

1.2 The pathophysiology of the renin-angiotensin system

1.2.1 Hypertension

As the renin-angiotensin system is intimately involved in the control of blood pressure and volume, it is not surprising that defects have been sought as contributing to the development of essential hypertension in humans. The efficacy of both ACE inhibitors and AII antagonists as antihypertensive therapy would seem to indicate a role for AII. Alterations in the renin-angiotensin-aldosterone system could partially explain the pathogenesis of malignant hypertension and many of the renin-dependent

forms of renovascular disease (Okamura *et al.* 1986; Dzau & Safar, 1988; Lifton, 1996). It has also been suggested that aberrant expression of AII in the blood vessels, the heart and the brain may contribute to the pathogenesis of essential hypertension (Ferrario, 1990). In hypertensive subjects, the vascular RAS is thought to be activated and there is evidence that reduced arterial compliance may also be a result of this (Dzau & Safar, 1988). This reduced compliance in conduit arteries is a predisposing factor for arteriosclerosis and could particularly affect essential hypertensives, even though the hypertension does not derive from an obvious defect in the RAS (Dzau & Safar, 1988).

Genetic polymorphisms in RAS components have been postulated as causes for essential hypertension (Peters, 1995); however, only one has been found. A single nucleotide mutation in the type 1 angiotensin II receptor (A1166→C) has been found at a higher frequency in hypertensive subjects (Bonnardeaux *et al.* 1994), implying that this common variant of the AT_1 receptor may impart a small effect on blood pressure. Linkages have been found for polymorphisms in RAS components and primary hypertension, pre-eclampsia, cardiac hypertrophy and myocardial infarction. A GT repeat 3′ of the transcription start site in the angiotensinogen gene has been linked to some forms of primary hypertension (Caulfield *et al.* 1994); the same gene has a second polymorphism involving a single amino acid substitution which associates with a greater tendency to pre-eclampsia (Ward *et al.* 1993). No defects linked to cardiovascular diseases have been found in the human renin gene. An insertion/deletion polymorphism in the ACE gene has been linked to both increased risk of myocardial infarction (DD genotype; (Cambien *et al.* 1992)) and left ventricular hypertrophy (Schunkert *et al.* 1994).

There is also a class of essential hypertensives called 'non-modulators' who display abnormal interactions of AII with the adrenal and sodium sensitivity of blood pressure (Williams & Hollenberg, 1991) in whom an

enhanced local RAS is suspected; correction of this defect with ACE inhibitors is very efficacious.

Finally, hypertension induced by glucocorticoids could be mediated by alterations in the renin-angiotensin-system. Increased levels of glucocorticoids have been shown to upregulate AII receptors in the vasculature *in vivo* and *in vitro* (Sato *et al.* 1994; Guo *et al.* 1995); more AII receptors leads to the possibility of increased AII action, causing vasoconstriction and increased blood pressure. Elevated plasma concentrations of glucocorticoids can also increase hepatic production of angiotensinogen and thus increase plasma AII levels, compounding the effect (Guo *et al.* 1995); glucocorticoids have also been shown to increase ACE activity (Mendelsohn *et al.* 1982).

1.2.2 Heart failure

Abnormal functioning of the renin-angiotensin system has also been implicated in other cardiovascular diseases. Again, this has been suspected by the better-than-expected recovery observed in ACE inhibitor therapy, and it is the growth factor properties of AII that are implicated. Abnormal activation of AII, and particularly the cardiac RAS, appears to be involved in cardiac and ventricular remodelling after infarction, cardiac hypertrophy and restenosis after angioplasty (Griendling *et al.* 1993b), as well as initiation of neointimal lesions due to disturbances of the cell growth/cell death equilibrium (Gibbons & Dzau, 1996). Treatment with ACE inhibitors slows progression of these conditions or even prevents them where other equally antihypertensive agents do not. These drugs therefore have a cardioprotective effect (Lindpaintner & Ganten, 1991), although part of this effect is thought to be due to inhibition of bradykinin metabolism. ACE has been shown to be activated in human heart failure, and this activation is thought to play a large role in cardiovascular pathophysiology (Paul *et al.* 1995).

Congestive heart failure is another disease in which overactivity of the renin-angiotensin system is implicated (Lindpaintner & Ganten, 1991). Increases in AII can lead to increases in heart overload (Dzau & Safar, 1988), which increases heart wall stress, and aggravation of heart failure. The cardiac RAS is also reported to be upregulated in number in congestive heart failure (Eiskjaer *et al.* 1992).

1.2.3 Vascular under-reactivity; renal disease

In both diabetes and cirrhosis of the liver, there is a peripheral vascular under-reactivity to angiotensin II. In the former case, this is thought to be due to the fact that glucose induces a downregulation of AII receptors in the vasculature (Williams *et al.* 1992); the explanation for the effect seen in cirrhosis is not so clear, but may be due to a decrease in affinity of the receptors or a lack of expression (Ryan *et al.* 1993). The mitogenic activation of AII in certain renal cell types, including glomerular endothelial cells, could also be important in the pathogenesis of glomerulonephritis (Wolf *et al.* 1996).

It is therefore clear that abnormalities in the renin-angiotensin system, whether in the genes or their expression, functioning or regulation, or at the local or systemic level, may have a major effect on many pathological states, and that further exploration of the system and its modulation could lead to more specific and beneficial therapies being found for some of the conditions described above.

1.3 Target tissues for Angiotensin II

1.3.1 The adrenal gland : structure, function and importance of AII

The healthy human adult has two adrenal glands, situated just above the kidneys and weighing 4-5g each. Both glands are similar in size, with slight variations in shape. The gland consists of a connective tissue capsule surrounding a dark cortex and a lighter inner medulla. The cortex is derived from mesodermal cells while the medulla is derived from immigrant neuroectodermal cells. The medulla comprises about 10% of the gland and its function is to synthesise, store and secrete catecholamines. The function of the cortex is to synthesise and secrete adrenal steroid hormones. It is the cortex that is preferentially acted upon by AII, and therefore this section will focus upon cortical structure and function.

1.3.1.1 Adrenocortical zonation

The adrenal cortex is further subdivided into three zones, shown by the diagram in figure 1.2. The name of each zone relates to the morphological appearance of its constituent cells. Thus the outer zona glomerulosa cells give the appearance of bundles, being succeeded by the column-like structures of the zona fasciculata and then by the zona reticularis, where the cells form a net-like structure.

The three zones differ functionally as well as morphologically, each containing the correct enzymes to synthesise a particular class of steroids. The zg, which synthesises mineralocorticoids, is the only zone containing aldosterone synthase, which catalyses the terminal step in aldosterone synthesis (see Figure 1.3). 11β-hydroxylase, required for glucocorticoid biosynthesis, is only present in the zf and zr.

The origin and control of zonation can be explained by two main hypotheses. The zonal hypothesis (Swann, 1940) proposes that each zone has a separate set of progenitor cells. The width of each zone can be controlled

by trophic hormones: increased AII levels increase zg width while high ACTH increases zf width at the expense of the zg.

Another hypothesis relies upon the observation that most cell division in the adrenal cortex occurs in the zg, and most cell death (by apoptosis (Wyllie *et al.* 1973)) in the zr. This cell migration hypothesis proposes that a common layer of stem cells, located either just beneath the capsule or between the zg and zf, divides continually, producing inward migration of cells through the cortex. How the cells adopt different phenotypes is unknown; either age or the steroid gradient theory of Hornsby (in (Neville & O'Hare, 1982), in which, by exposure to high steroid levels and pseudosubstrates, adrenocortical cells may change phenotype) may be responsible. Radial striped patterns of transgene expression have been observed (Morley *et al.* 1996), consistent with this hypothesis. Again, zone width would most probably be under the control of the trophic hormones, AII and ACTH.

1.3.1.2 Structural features of the adrenal gland

An important feature of the adrenal gland, which also contributes to its function, is its vasculature. It is involved in corticomedullary interactions and possibly functional zonation and growth of the gland. The right adrenal is supplied directly from the aorta, while the left adrenal artery is a tributary of the renal artery. These both enter the capsule to give an extensive arterial plexus, from which some arterioles pass directly to the medulla. The cortical blood supply differs : the zonae fasciculata and reticularis are supplied by only a capillary network; blood drains down to the medulla first via the zona glomerulosa, then the two inner zones, before being collected in the large central vein.

Until recently, it was not thought that the adrenal cortex was directly innervated. However, it is now apparent that not only is there innervation of the adrenal cortex from two different sources, both independent of and

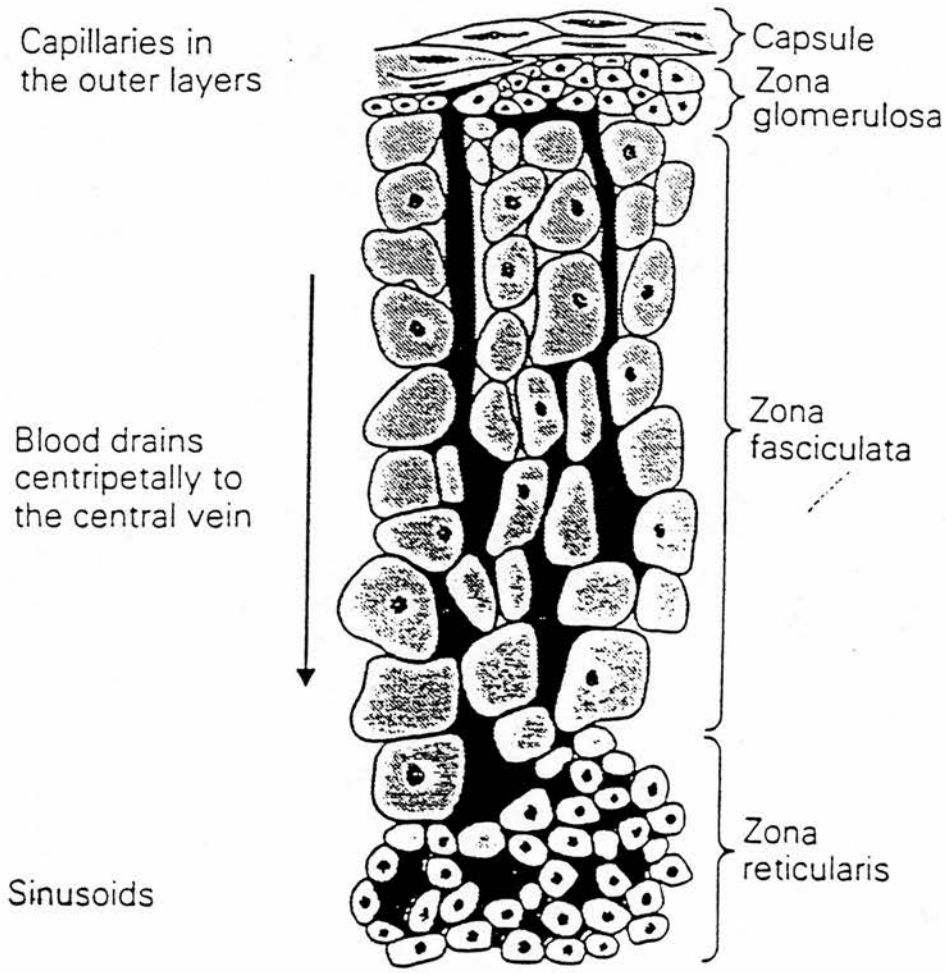


Figure 1.2

The morphological zonation of the adrenal cortex, showing the three types of cells and their particular structural arrangement.

regulated by the splanchnic nerve, there is also neural supply from the medulla to the cortex (Vinson *et al.* 1994). The neurotransmitters used in adrenocortical innervation include noradrenaline, vasoactive intestinal peptide, neuropeptide Y and acetylcholine (Clyne *et al.* 1994; Vinson *et al.* 1994), all of which can affect steroidogenesis in the adrenal cortex. The adrenal medulla is innervated by preganglionic fibres of the sympathetic nervous system. Each fibre innervates a group of cells to form a functional unit; separate units have separate synapses.

1.3.1.3 Functions of the adrenal cortex

The adrenal cortex secretes over 20 steroid hormones, divided into three classes : mineralocorticoids, glucocorticoids and adrenal androgens (Brooks, 1979). Table 1.1 shows the most common of these and their relative abundances, while figure 1.3 shows the steroid synthetic pathway operative in the human adrenal cortex, including the intracellular location of the enzymes. The precursor molecule for all steroid hormones is cholesterol, and the transport of cholesterol to the inner mitochondrial membrane, facilitated by StAR protein (Stocco & Clark, 1996) is the main site of control of steroidogenesis by pituitary hormones e.g. ACTH. Later steps in the pathway are controlled by other mechanisms, e.g. AII upregulates aldosterone synthase, allowing more aldosterone to be produced.

Most of the steroidogenic enzymes are members of the cytochrome P450 family of oxidases. Four distinct enzymes are involved in adrenocortical steroidogenesis :

- $P450_{scc}$ (side chain cleavage) mediates the conversion of cholesterol to pregnenolone
- $P450_{c11}$ mediates 11β-hydroxylation (e.g. 11-deoxycortisol to cortisol)
- $P450_{c17}$ mediates both 17α-hydroxylation and 17,20-lyase activity
- aldosterone synthase mediates the terminal steps in aldosterone biosynthesis

$P450_{c11}$ catalyses the final step in glucocorticoid synthesis, while aldosterone synthase catalyses the terminal step in mineralocorticoid synthesis; the two enzymes are therefore complementary. Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase. The sites of and chemical reactions catalysed by these enzymes are shown in Figure 1.3. For the enzymes capable of catalysing more than one reaction, e.g. $P450_{c17}$, the direction of the reaction depends upon the relative levels of substrate and product in the adrenocortical cell, and on the nature of the steroidogenic stimulus.

1.3.1.3.1 Steroid hormones

Mineralocorticoids affect the body's electrolyte composition by increasing sodium retention and increasing potassium excretion in the kidney. The principal hormone of this class in humans is aldosterone, although deoxycorticosterone also displays some mineralocorticoid activity. Aldosterone is synthesised uniquely in the zona glomerulosa; its secretion is under the control of the RAS as previously described (section 1.1.2), but increases in potassium concentration also stimulate its production, as do ACTH and serotonin. Potassium in fact interacts with AII in aldosterone production, an equilibrium which can become disturbed by drugs used to treat hypertension and heart failure (Vallotton *et al.* 1995). Deoxycorticosterone, however, is mainly produced in the zona fasciculata, thus mineralocorticoid secretion is not the sole province of the zona glomerulosa.

The zona fasciculata secretes most of the body's glucocorticoids. These have major effects on the metabolism of carbohydrates, fats and proteins, raising the concentration of glucose in the blood, and have an important anti-inflammatory action. The main hormone of this class in humans and cattle is cortisol; in rats the steroid pathway differs and the major product of glucocorticoid biosynthesis is corticosterone due to the absence of $P450_{c17}$

(see Fig. 1.3). Secretion of cortisol and corticosterone is under the principal control of ACTH from the anterior pituitary and therefore follows its basal circadian variation. This control functions as part of the hypothalamo-pituitary-adrenal axis, in which increased cortisol levels decrease CRF levels, leading to decreased ACTH secretion from the pituitary. All cortisol is produced in the zona fasciculata, as is half the corticosterone, the remaining half being synthesised in the zona glomerulosa and draining to the zona fasciculata to be 17-hydroxylated (Neville & O'Hare, 1982).

The adrenal androgens, synthesised predominantly in the zona reticularis of most mammals (with the exception of murine species), are the precursors to the sex steroids (androgens and oestrogens). The main compounds secreted are dehydroepiandrosterone (DHEA) and its sulphate, androstenedione and 11β-hydroxyandrostenedione. These are only weakly androgenic but are secreted in large amounts (DHEA : 20μg/day) so their effect may be significant. They pass through the circulation to the sex organs where they are activated to the sex hormones oestrogen and testosterone (although some activation may occur peripherally). Secretion of the adrenal androgens is again principally under the control of ACTH although other non-ACTH factors (McKenna *et al.* 1996), for example the putative pituitary hormone CASH, may also play a part. (CASH = cortical androgen stimulating hormone)

1.3.1.4 Importance of AII in the adrenal gland

The primary role of angiotensin II in the adrenal gland is the control of aldosterone synthesis and secretion (via the RAS). However, it has been demonstrated (see section 1.1.3) that AII can also stimulate cortisol secretion in the human (McKenna *et al.* 1978), bovine (Clyne *et al.* 1993) and porcine (Breidert *et al.* 1996) adrenal cortex, suggesting that this stimulus also plays a part in the inner zone. It is also known that AII can facilitate sympathetic neurotransmission, and secretion of steroids can be affected by neurotransmitters.

The existence of a local adrenal RAS also presents the possibility of both auto- and endocrine effects of AII. Angiotensin II is therefore an important influence in the control of steroidogenesis in the adrenal cortex. As AII can also stimulate the release of catecholamines from the adrenal medulla, raised or lowered levels of RAS activity could have profound effects on the correct functioning of the gland.

1.3.2 The resistance arteries : structure, function and importance of AII

All arteries, except the pulmonary artery, transport oxygenated blood away from the heart to capillary beds in the various organs and tissues of the body. Large conduit arteries (aorta, carotid, pulmonary, femoral etc) lead into medium sized muscular arteries (mesenteric, splenic, tibial etc : most of the body's arteries are of this group). These then feed into arterioles which have a lumen diameter of 30μm, decreasing to 8μm in precapillary arteries. The arterioles then connect with veins via capillary beds or, in some cases, anastomoses (Amenta, 1991).

Arterial walls consist of the following layers, which vary slightly in composition according to the calibre of the artery :

tunica interna (intima)

This is the internal coat of the artery facing the lumen and consists of an inner simple squamous endothelium, a stratum subendotheliale (a layer of loose connective tissue) and an internal elastic membrane (a network of elastic connective tissue fibres).

tunica media

A middle layer of circularly oriented smooth myocytes, with some elastic fibres, separated from the tunica externa by an external elastic membrane.

Steroid	Relative activity in assay				
	MR	Na^+	GLY	GR	AI
Aldosterone	1	1	0.15	1	—
DOC	0.8	0.03	0.02	1	—
Corticosterone	0.2	0.004	0.36	2	0.3
Cortisol	0.1	0.001	1	1	1
18-OH-DOC	0.015	0.004	—	0.02	—
Dexamethasone *	0.05	0.001	17–250	10	25–169
9α-fluorocortisol *	1*	0.15	6–13	10*	8–15

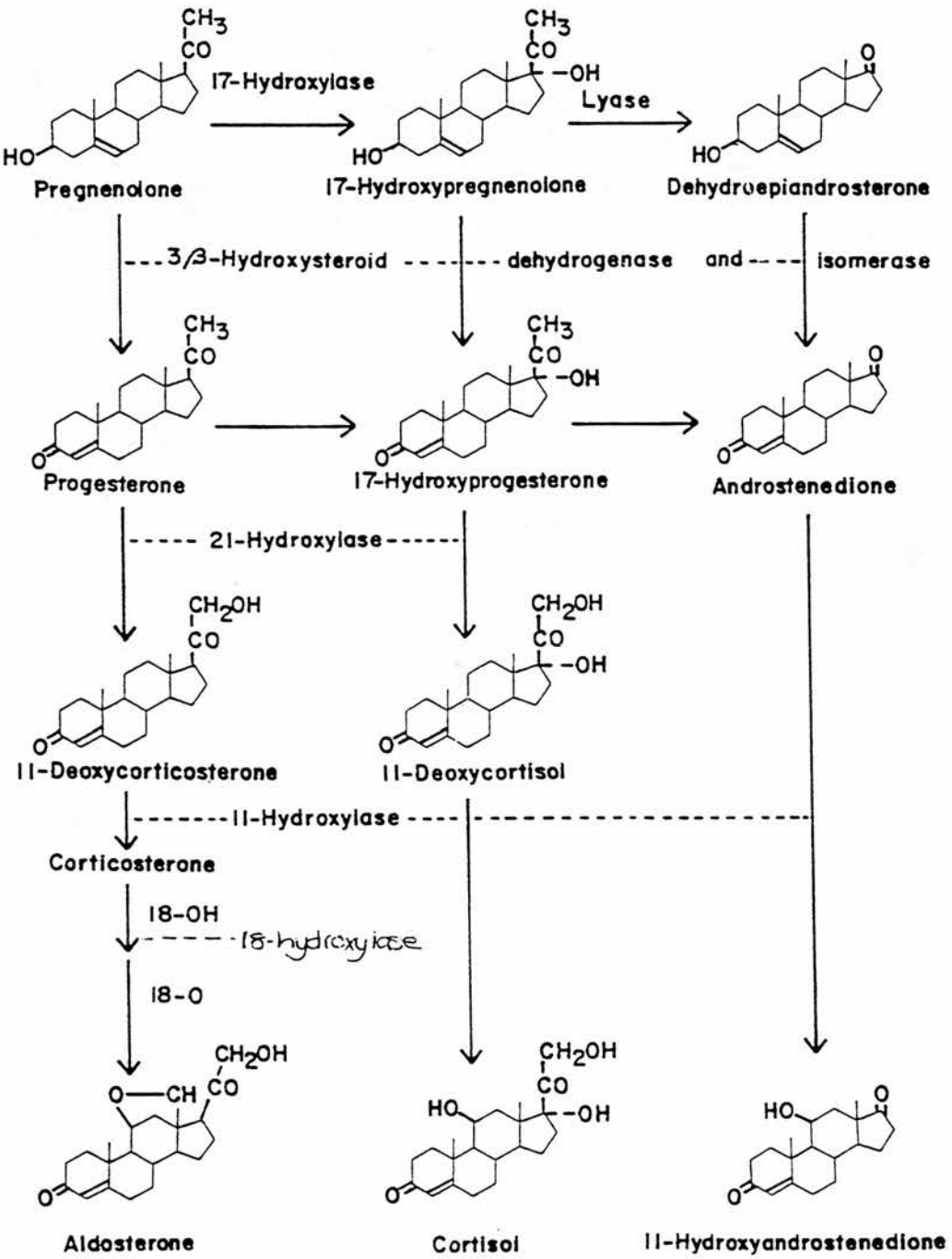
MR = mineralocorticoid receptor assay based on competition to ^{3}H-aldosterone binding sites in rat kidney
Na^+ = bioassay, normally based on change of urinary Na^+/K^+ in the adrenalectomized rat
GLY = bioassay, based on glycogen deposition in the adrenalectomized rat liver
GR = glucocorticoid receptor assay, based on competition with ^{3}H-dexamethasone binding sites in rat or sheep(*) kidney
AI = bioassay based on anti-inflammatory activity

Table 1.1

Activities of corticosteroids. The biological activities of four endogenous and two artificial (*) steroids in various assays are given in the table above (reprinted from Vinson, 1993).

Figure 1.3

The steroid biosynthetic pathway. All adrenocortical steroids are synthesised from the common precursor pregnenolone. The three classes of steroids are secreted by the three zones of the cortex as detailed in the text. All enzymes except $P450_{scc}$, $P450_{c11}$ and aldosterone synthase are found in the microsomal compartment, the remainder are mitochondrial.


CH3
CO
17-Hydroxylase
HO
Pregnenolone
CH3
CO
-OH
HO
17-Hydroxypregnenolone
Lyase
O
HO
Dehydroepiandrosterone
---3/β-Hydroxysteroid ---------- dehydrogenase
and ---- isomerase
CH3
CO
Progesterone
CH3
CO
-OH
17-Hydroxyprogesterone
O
Androstenedione
----- 21-Hydroxylase ------
CH2OH
CO
11-Deoxycorticosterone
CH2OH
CO
-OH
11-Deoxycortisol
-------11-Hydroxylase ----
Corticosterone
18-OH
- - - - - 18-hydroxylase
18-O
O—CH
CO
Aldosterone
CH2OH
CO
HO
-OH
Cortisol
HO
O
11-Hydroxyandrostenedione

tunica externa (adventitia)

The outermost coat of connective tissue.

Figure 1.4 shows the generalised structure of an artery (described in detail in Amenta, 1991).

In the arterioles, the intima consists of endothelial cells resting on a basement membrane, with a pronounced internal elastic membrane. The tunica media contains 1-3 layers of spiralling smooth myocytes, while fibroblasts and collagen fibres comprise the tunica externa.

The muscular arteries are thicker as they have to conduct blood at higher pressure than the arterioles, whose main role is to provide and modulate peripheral resistance. Thus the intima is thicker, with the endothelial cells sending processes into the media, and a more prominent stratum subendotheliale. This layer thickens where the arteries bifurcate, providing a better control of blood flow. The media is also thicker, and the outer tunica externa consists of loose connective tissue.

The large arteries are more elastic, to enable them to withstand the very high pressures of blood flowing from the heart, and therefore elastic fibres predominate over myocytes in the media, which is again thicker than in the muscular arteries. The intima is similar to that of the muscular arteries, but the externa is tightly encircled with collagen fibres.

Some arteries are of mixed type, intermediate between the elastic and muscular arteries (e.g. the renal and coeliac arteries) or specialised to adapt to specific situations or locations (e.g. the umbilical and coronary arteries).

The function of the vasculature relevant to this study is the provision and modulation of resistance to blood flow. Resistance increases with decreasing luminal diameter. Therefore, in the systemic circulation (i.e. excluding the pulmonary circulation), the majority of the resistance lies in the arterioles. This resistance offered by blood vessels to blood flow is termed peripheral resistance.

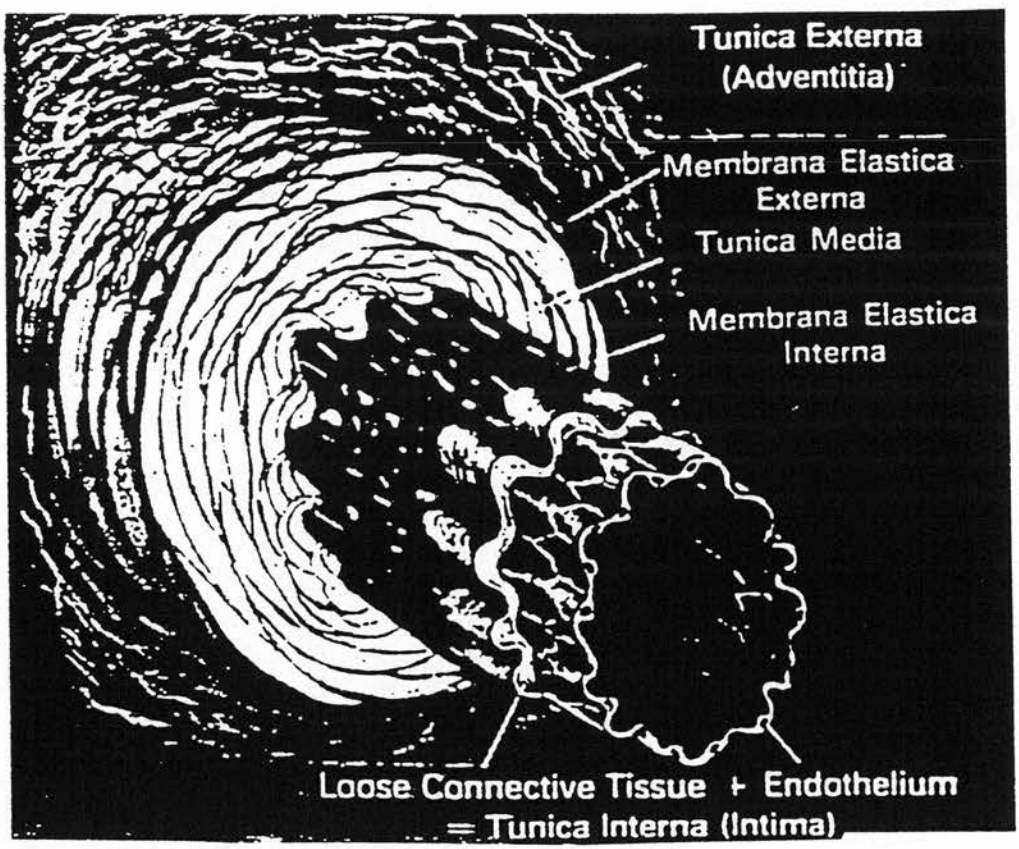


Figure 1.4

Generalised structure of the arterial wall, showing the different layers and their constituents (taken from Amenta, 1991).

The peripheral resistance can be changed by a constriction or dilatation of the blood vessels, most commonly in the arterioles but sometimes in the muscular arteries. The smooth muscle in the arterial wall is in a state of partial contraction due to continuous sympathetic nerve activity. This state is known as vasoconstrictor tone and originates in the vasomotor centre in the medulla oblongata. Vasomotor centre activity can be modulated by baroreceptor reflexes (from the aortic arch and carotid sinus), which, when stimulated, inhibit the activity of the centre. The action of the vasomotor centre, however, is limited compared with that of systemic or local vasoconstrictors, such as angiotensin II, or vasodilators.

Angiotensin II is an important agent in arterial smooth muscle function, acting preferentially in the precapillary arterioles to increase peripheral resistance and raise blood pressure. Inadequate blood flow to the kidney stimulates renin release. This initiates the RAS cascade as described previously (section 1.1.2), ending in the formation of AII, which interacts with its type 1 receptors to induce widespread vasoconstriction. This may be due to the phosphorylation of the smooth muscle myosin light chain, enabling contraction to occur (Mendelsohn, 1985). When the myocytes contract, the vessel lumen narrows and tension is established in the elastic membranes, which become wavy. This narrowing of lumen diameter results in an increase of blood pressure in the vessel. Contraction is more prevalent in the resistance arterioles for two reasons : firstly, there are more AT_1 receptors in these vessels, and secondly, the large arteries have less capacity to contract *in vivo* due to the greater pressure to which they are subjected.

1.4 Angiotensin II receptors

1.4.1 Discovery and historical perspective

The presence and location of specific binding sites for angiotensin II in the adrenal gland was first shown in 1964 by ^{3}H-AII ligand binding

(Bumpus *et al.* 1964). Goodfriend and Lin (1970) showed, using ^{125}I-AII, that these binding sites in rat adrenal cortex exhibited the characteristics of receptors : the binding was specific, reversible, saturable and of high affinity. Specific AII binding sites were later demonstrated in rabbit and guinea pig aorta (Baudouin *et al.* 1972; le Morvan & Palaic, 1975), rat uterus (Devynck *et al.* 1976), and rat, rabbit and bovine adrenal cortex (Glossmann *et al.* 1973; Gurchinoff *et al.* 1975; Forget & Heisler, 1976). AII receptors have since been demonstrated in renal glomeruli (Bennett & Snyder, 1976) and whole brain (Sraer *et al.* 1974). These studies showed that the receptor was located in the plasma membrane of the target organ cells. Other investigators, however, reported binding sites in isolated mitochondria (cited in Devynck & Meyer, 1978) and nuclear membranes (Robertson & Khairallah, 1971).

It is now known that AII receptors are integral membrane proteins which are internalised and recycled (Hunyady *et al.* 1994b), although it was shown recently, using radioligand binding , that there are AII receptors in the nucleus (Jimenez *et al.* 1994). The binding affinity of AII for its receptors in most target tissues was found to be in the range of 10^{10} - 10^{9}M (Lin & Goodfriend, 1970); this would imply half-saturation of the receptors at concentrations of AII of 0.1-1nM, whereas basal blood levels of AII are much lower (0.025nM). Such discrepancies are common for peptide hormones, however, and reflect the ability of many target organs to be activated by only fractional occupancy of receptors (Catt & Aguilera, 1980).

Later studies were able to locate AII receptors more precisely, for example Douglas et al (cited in Devynck & Meyer, 1978) showed that in the rat adrenal, AII receptors were more concentrated in the zona glomerulosa, with fewer in the zona fasciculata. In the bovine species, though, the difference in distribution is not so great, as there are more receptors in the zona fasciculata than observed in the rat (Catt & Aguilera, 1980). The majority of sites corresponded to known areas of AII action, including the pituitary gland, subfornical organ and area postrema, whereas some

investigators reported AII binding sites in less expected locations, for example in platelets (Mendelsohn, 1985).

The low numbers of AII receptors in target tissue cell membranes and their relative instability made isolation extremely difficult, although characterisation of AII receptors in dog adrenal cortex and uterus by photoaffinity labelling (Capponi & Catt, 1980) yielded receptors of MW 65 and 68kDa respectively. It was not until after the receptor was cloned in the early 1990s that the structure was clearly elucidated.

1.4.2 Heterogeneity of AII receptors

The studies described above, conducted in the 1970s and early 1980s, suggested the possibility of heterogeneity of angiotensin II binding sites or receptors. The main arguments for this were as follows :

- Altering the salt or potassium status of rats had reciprocal effects on receptor number and/or affinity in the adrenal gland and vascular smooth muscle (Douglas, 1979; Aguilera *et al.* 1980; Aguilera & Catt, 1981). For example, sodium depletion led to an increase in receptor number in the adrenal cortex, but a decrease in vascular smooth muscle (Aguilera *et al.* 1980).

- Studies by some groups in a variety of target organs, including adrenal, liver and smooth muscle, indicated only a single population of receptors when ligand binding data was fitted to a Scatchard plot (reviewed in Devynck & Meyer, 1978). However, other groups suggested a heterogeneous population (Wright *et al.* 1983; Gunther, 1984; Hausdorff *et al.* 1987). Indeed, Gunther's group published the existence of two classes of AII binding sites in rat hepatocytes : one high and one low affinity.

- In Gunther's study, only the low affinity site was susceptible to dithiothreitol, which inhibited ^{125}I-AII-binding (Gunther, 1984).

- In the early 1980s, it became apparent that AII could work through two different signal transduction pathways (IP_3/Ca^{2+} and adenylate cyclase/cAMP : see section 1.4.3.1). This was used as further evidence that AII receptors could belong to more than one class (Balla *et al.* 1991).

- At this time, the only AII antagonists available to researchers were peptide analogues of AII, including the most widely used, saralasin ([sar^1-val^5-ala^8]-AII) and sarile ([sar^1-ile^8]-AII). These were not able to distinguish between any potential subclasses of AII receptors. However, the potency order of these analogues and antagonists differed between tissues. (TIMMERMANS *et al.* 1993)

- The heptapeptide breakdown product of AII, AIII, was also shown to bind with differing affinities to the two potential classes of receptor (Griendling *et al.* 1993a). AIII bound with equal affinity to the 'low-affinity' site, and less potently to the 'high-affinity' site.

Discrimination between two classes of angiotensin II receptors became possible following the development of non-peptide antagonists. In 1989, Whitebread *et al.* (1989) and Chiu *et al.* (1989) published reports of two classes of non-peptide antagonists, the biphenylimidazoles, exemplified by DuP753 (losartan), and the tetrahydroimidazopyridines, exemplified by PD123319 and PD123177, which could discriminate between two subtypes of AII receptor. Whitebread's group divided the AII receptors into type A, found in uterus and blocked by CGP42112A (a compound related to PD123177), and type B, found in vascular smooth muscle, blocked by Ex89 (precursor compound to DuP753), binding to which sites was abolished by dithiothreitol. Chiu's group, who studied the rat adrenal gland, also identified two subtypes. They named them AII-1, found mainly in the cortex and blocked by DuP753, and AII-2, found almost exclusively in the medulla and blocked by EXP655 (a PD-like compound). A combination of the two

non-peptide antagonists abolished all ^{125}I-AII binding in the rat adrenal gland.

Chang & Lotti (1989) further demonstrated that it was the type B or AII-1 (now named AT_1) receptor which mediated the known physiological actions of AII. The presence of two subtypes of AII receptors has since been demonstrated in many target tissues of AII (reviewed in Timmermans *et al.* 1993).

There is a wide species variation in the tissue distribution of angiotensin II receptors. In the rat adrenal, 20% of binding in the cortex is to the AT_2 subtype, whereas in the bovine adrenal cortex the proportion of AII-2 receptors is negligible. In vascular smooth muscle, some species (rat) have almost entirely AT_1 receptors, while others (rabbit, dog) have a mixed population. The AT_1 subtype is mainly found in the adrenal cortex, vascular smooth muscle, liver, kidney, heart, pituitary and uterus while the AT_2 receptor predominates in the adrenal medulla and brain. AT_2 receptors are also expressed at a high level throughout foetal development, with levels declining towards birth (Timmermans *et al.* 1993).

Another AII receptor subtype has also been reported. Sandberg *et al.* (1992) showed by molecular methods the presence in rat adrenals and pituitary gland of a type 1-like receptor which was not identical to that found by previous investigators and was thus termed the AT_3 receptor.

Receptors have also been discovered for a smaller homologue of AII : AII(3-8), also known as AIV. Harding *et al* first proposed such binding sites (Swanson *et al.* 1992) in guinea pig hippocampus; distinct from AII receptors, neither AII or AIII could displace AIV from these binding sites. The same group later published a report of AIV binding sites in other tissues (Harding et al, 1994) : aorta, brain, heart, kidney, liver lung and adrenal cortex of a number of species, including bovine and guinea pig. Since AIV is reported to have effects antagonistic to AII, the finding of these receptors is likely to have profound implications for understanding the renin-angiotensin system (Harding *et al.* 1994).

The presence of multiple AII receptors, and the possibility that AII may have additional physiological effects to those previously categorised, suggests that the true physiological significance of AII receptor subtypes is not yet fully understood.

1.4.3 Cloning of angiotensin II receptors

The molecular cloning of AII receptors described in this section, as well as yielding important structural information about both AT_1 and AT_2 receptors, has led to subdivision of the AT_1 subclass. In rodents, the AT_1 receptor has two forms, AT_{1a} and AT_{1b}; a third subtype, AT_{1c}, has also been proposed. As yet, additional AT_1 subtypes have yet to be described for bovine and human species.

1.4.3.1 Cloning of the AT_1 receptor

The type 1 angiotensin II receptor was first cloned in 1991 by two groups simultaneously from bovine adrenocortical cells (Sasaki *et al.* 1991) and rat vascular tissues (Murphy *et al.* 1991), using expression cloning techniques. Both receptor clones contained an open reading frame of 1,077bp, encoding a protein of 359 amino acids, yielding a predicted 41kDa protein. The entire coding region, a portion of the 5′ untranslated region and the 3′ untranslated region are all contained within a single exon (Bernstein & Alexander, 1992). Hydropathy analysis revealed that the receptor belonged to the seven-transmembrane region class of G-protein coupled receptors, and the presence of one cysteine residue in each of the four extracellular loops indicated that these could form disulphide bridges, providing a site for ligand binding. There are 3 potential sites for N-glycosylation (one in the N-terminal extracellular region and two in the third extracellular loop), which would account for the higher MW of 65kDa reported for the AT_1 receptor

(Griendling *et al.* 1993a). The second and C-terminal cytoplasmic domains contain serine and threonine residues which may be important in regulation of receptor activity by phosphorylation. There was only a 20-30% sequence identity with other known G-protein-coupled receptors, and also with the *mas* oncogene, which had previously been postulated to encode an AII receptor (Jackson *et al.* 1988). A pictorial representation of the rat vascular AT_1 receptor is shown in Figure 1.5.

The bovine adrenal receptor gene encoded an mRNA of 3.3kb, which was expressed in kidney as well as both adrenal cortex and medulla. When expressed in cos-7 cells, the protein displayed the pharmacological characteristics of an AT_1 receptor. The vascular receptor, isolated from a rat aortic cDNA library, contained the same size open reading frame as in the bovine adrenal clone, but two transcripts of different size were found in various rat tissues. A transcript of 3.5kb was observed in liver, kidney, adrenal, lung and very strongly in vsmc. A smaller, 2.3kb transcript was present at a much higher abundance in all the above tissues than the larger transcript, and was additionally detected in aorta, uterus, ovary, heart and spleen. These two transcripts may represent either alternative splicing of the gene, alternative processing of a common RNA precursor, or two highly similar products from the same gene. Later analysis of the cloned human AT_1 receptor (Curnow *et al.* 1995) indicates that the first possibility is the correct one. The greatest number of differences between the rat and bovine receptors was in the carboxyl terminus (Bernstein & Alexander, 1992).

Shortly after this, other investigators reported the cloning of the AT_1 receptor from other species, and also the further discovery of a second AT_1 receptor subtype in the rat and, later, in mouse (Iwai *et al.* 1991; Iwai & Inagami, 1992a; Yoshida *et al.* 1992).

Iwai *et al.* (1991) cloned an AT_1 receptor identical to that cloned by Murphy et al, from a rat kidney cDNA library. Soon afterwards, a second AT_1-type receptor was cloned, from rat adrenal (Iwai & Inagami, 1992a) and pituitary (Kakar *et al.* 1992). The vascular and kidney subtype was classified

as AT_{1a}, and the adrenal and pituitary subtype AT_{1b}. The two subtypes showed 96% homology at the amino acid level, and both encoded a 359 amino acid, seven-transmembrane region type of receptor, with virtually identical N-glycosylation sites and cysteine residues. Pharmacologically (when transiently expressed in cos-7 cells) both displayed the characteristics of AT_1 receptors and were indistinguishable using a wide range of AT_1-specific antagonists (Balmforth *et al.* 1994), although they were the products of distinct genes. The 5′ and 3′ untranslated regions of the mRNAs for the two AT_1 receptors displayed very little homology, raising the possibility that the two genes could be regulated differently (Bernstein & Alexander, 1992), (see 1.4.5). Differences in the coding regions of the two types were concentrated in the carboxy termini of the receptors, which could be important in differential regulation, as this is this region where regulatory phosphorylation occurs (Griendling *et al.* 1993a). The mRNA for the rat AT_{1a} subtype was found preferentially in the vasculature, lung and ovary, while that for the AT_{1b} was found in the adrenal, anterior pituitary and the uterus, and both were present to the same extent in spleen, liver and kidney (Kakar et al 1992). A later study by Gasc *et al.* (1994) used in situ hybridisation to localise the two rat subtypes by cell type. This showed some differences in the pattern of expression, for example in the pituitary, the mRNA for AT_{1a} was expressed in the intermediate and posterior lobes, and that for AT_{1b} in the anterior lobe.

The AT_{1b} receptor was found to be identical to the AT_3 subtype cloned by Sandberg *et al.* (1992). A third AT_1 receptor subtype has also been reported in rats, the AT_{1c} receptor cloned by Hahn *et al.* (1993). This receptor was detected in brain, kidney, liver, lung, spleen and abundantly in vsmc and mesangial cells, and has 90% homology at the nucleic acid level with the AT_{1a} and 82% with the AT_{1b} subtype. The significance of this third AT_1 subtype is not yet clear.

The human AT_1 receptor gene has also been cloned (Takayanagi *et al.* 1992; Furuta *et al.* 1992) but, like the bovine receptor, no AT_1 subtypes have

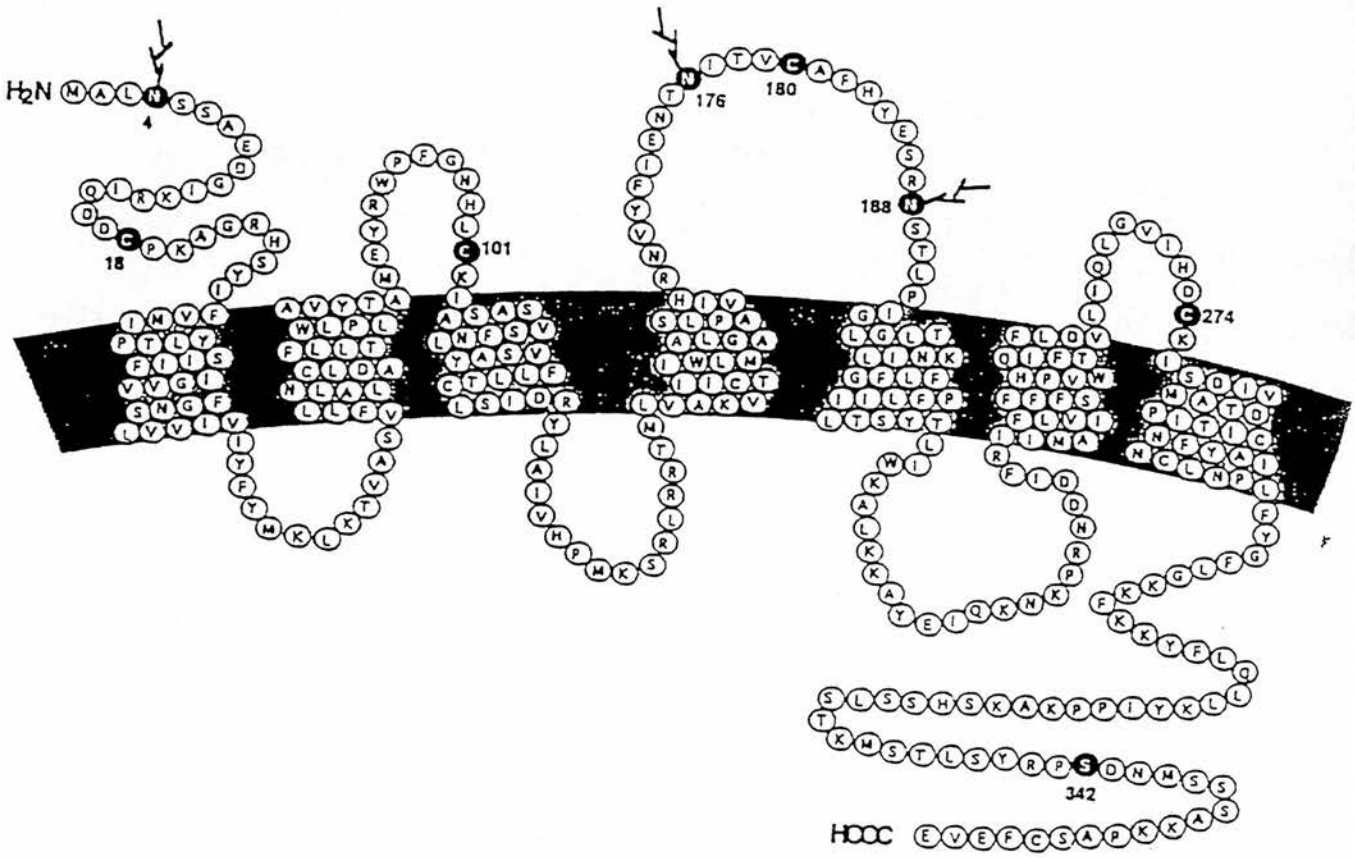


Figure 1.5

Representation of the structure of the rat vascular AT_1 receptor (Griendling *et al.* 1993a). It is a seven-transmembrane region G-protein coupled receptor. The branched regions indicate N-glycosylation sites, and the dark cysteine residues in all probability form two disulphide bonds important for ligand recognition.

been demonstrated to date. It also encodes a 359-amino acid protein with an open reading frame of 1,077 bp, and exhibits 95% homology with the bovine receptor and 94% with the rat AT_{1a} receptor. A 2.3kb AT_1 receptor transcript can be detected in the human liver, lung and adrenal. Work done by Curnow *et al.* (1995) indicates, however, that although there is only one subtype, alternative splicing of the mRNA occurs yielding two transcript sizes, a situation comparable with the rat AT_{1a} mRNA.

Analysis of the AT_1 receptor gene by Yoshida *et al.* (1992) has suggested that there are at least two genes for AT_1 receptors in the rat and mouse (which they cloned in this paper), but only one in bovine and human, and so far, this has proved to be the case.

1.4.3.2 Important functional elements of the AT_1 receptor

The amino acid sequences of AT_1 receptors from bovine, rat and human tissues are highly conserved (Inagami *et al.* 1992). Site-directed mutagenesis has been used to elucidate which sequences of amino acids are important for receptor activation and internalisation.

Four cysteine residues are present in the four extracellular loops of the receptor, one cysteine in each loop (Griendling *et al.* 1993a). These probably form two disulphide bridges, which would provide a ligand-binding domain. The presence of such disulphide bridges would explain the sensitivity of the AT_1 receptor to dithiothreitol.

Like other peptide hormone receptors, the AT_1 receptor is internalised after complexing with its ligand. Certain residues in the AT_1 receptor have been found to be vital for this process (Balmforth *et al.* 1995) : deletion of 41 amino acids from the carboxy terminus of the receptor sequence led to an impairment of receptor internalisation after binding to AII, but the signal transduction coupling was normal.

The critical nature of tyrosine residue 215 in G-protein coupling was elucidated by its substitution with phenylalanine, which resulted in a

mutant receptor with reduced affinity for AII analogues and no IP_3 responses after stimulation with AII (Hunyady *et al.* 1995) This residue was also found to be important for internalisation of the receptor, as this process was impaired in the mutant receptor. Recently, another tyrosine residue, tyrosine 302, was shown to be involved in mediating signal transduction but its mutation did not affect internalisation (Laporte *et al.* 1996).

Further site-directed mutagenesis experiments (Hunyady *et al.* 1994b) showed that the aspartate residue 74 is also extremely important for receptor activation, since its substitution with asparagine results in severely impaired G-protein coupling and IP_3 signalling, but normal ligand binding and internalisation kinetics. Mutation of this same residue to tyrosine (Hunyady *et al.* 1994a) resulted in greatly impaired internalisation, but signal transduction similar to the wild type receptor, while deletion of residues 215-220 severely impaired internalisation, and deletion of 221-226 resulted in no G-protein coupling. There are therefore different requirements for receptor internalisation and second messenger signalling, leading to the conclusion that activation of G-proteins and signal transduction is unlikely to be required for receptor internalisation, and the the two processes are distinct. This was confirmed by Kapas *et al.* (1994), and Vinson *et al.* (1995a), who used a monoclonal antibody to the AT_1 receptor to anchor the receptor in the cell membrane of rat zg cells, thus preventing internalisation, but permitting activation of PLC after stimulation with AII. PKC, however, was not activated under these circumstances, suggesting that internalisation is necessary for PKC activation to occur.

1.4.3.3 Cloning of the AT_2 receptor

Historically, study of the AT_2 receptor was hampered by the inability to assign a known physiological response or signal transduction system to this subtype. The AT_2 receptor cDNA was cloned simultaneously from a rat PC12W cell cDNA library (Kambayashi *et al.* 1993b) and a rat foetus

expression library (Mukoyama *et al.* 1993), both of which tissues are known to express high levels of AT_2 receptors. In each case the cDNA encoded a 363 amino acid protein with 34% homology to the rat AT_{1a} receptor. Hydropathy analysis showed that it belonged to the seven-transmembrane region G-protein coupled receptor family. It was most similar to the dopamine D_3 and somatostatin receptors, a class of receptors in which G-protein coupling exists but where there is no sensitivity to GTP analogues. The AT_2 receptor was shown to be coupled to a pertussis toxin sensitive protein (Mukoyama *et al.* 1993). Initial evidence (Kambayashi *et al.* 1993b) showed that the AT_2 receptor was coupled to a protein tyrosine phosphatase, and that stimulation of the receptor modulated the phosphatase activity. Further studies have confirmed this (Kambayashi *et al.* 1994; Yamada *et al.* 1996), as well as assigning a putative function for AT_2 receptors in mediating apoptosis. Both groups found N-glycosylation sites and also serine and threonine residues for possible regulatory phosphorylation. The mRNA transcript is 3.5kb in length, and is highly expressed in foetal tissues and in adult adrenal, brain and uterus.

The human AT_2 receptor, 92% identical to the rat sequence, has also been cloned (Martin *et al.* 1994), as has the mouse subtype (Nakajima *et al.* 1993). The human gene transcript is of a similar size to that of the rat gene, and is present in adult lung, heart, aorta and adrenal medulla, and highly expressed in foetal kidney and lung.

At the time of its cloning, little was known about the function of this receptor and its signal transduction, and therefore no work similar to that for the AT_1 receptor has been carried out in terms of site-directed mutagenesis. It is likely, though, that similar regions of importance will be uncovered in the near future.

1.4.4 Mechanism of AII receptor function

1.4.4.1 AT_1 receptors

The AT_1 receptor subtype, until very recently, was thought to mediate all the physiological actions of angiotensin II. In 1984, de Lean et al published evidence that the AII receptor in bovine adrenal zona glomerulosa interacted with a G-protein to stimulate the secretion of aldosterone (de Lean *et al.* 1984a). Most of the physiological responses to AII are now known to be mediated *via* the IP_3/Ca^{2+} signal transduction system (Vallotton, 1987). Some groups have also indicated that AII inhibited basal and ACTH-stimulated cAMP generation (Hausdorff *et al.* 1987), and that AII increased cAMP generation (Rainey *et al.* 1991). AII also stimulates 12-HETE, a lipoxygenase intermediate, through the AT_1 receptor (Natajaran *et al.* 1988; Stern *et al.* 1993). A recent report by Marrero *et al.* (1995) shows that the Jak-STAT tyrosine kinase pathway, commonly used by cytokines and thought to be purely an intracellular pathway, is also stimulated by AII through the AT_1 receptor in rat aortic smooth muscle cells. Phospholipase A_2 can also be stimulated by AII through the AT_1 receptor, although this may be secondary to PKC stimulation (Griendling *et al.* 1993a). Figure 1.6 shows a representation of the signal transduction pathways known to be activated by AII through the AT_1 receptor.

The steroidogenic response to AII is mediated through a pertussis toxin-insensitive G-protein (G_q) at low AII concentrations, but supramaximal concentrations of AII involve a pertussis toxin-sensitive G-protein, which mediates attenuation of the steroidogenic response (Griendling *et al.* 1993a). The sequence of events triggered by activation of the AT_1 receptor is identical in vascular smooth muscle and the adrenal cortex and consists of two successive phases (Griendling *et al.* 1993a). The first starts within seconds of exposure to AII and lasts for ~1 minute. There is a rapid activation of PLC, with concomitant generation of IP_3 and a rapid rise in intracellular calcium and activation of PKC. The second phase starts within 1

minute of exposure and lasts for up to half an hour. PLD is activated, leading to an accumulation of phosphatidic acid, which results in increasing DAG levels and maintains PKC activation. This is accompanied by a sustained influx of extracellular calcium, which may participate in intracellular calcium oscillations. This second phase, unlike the first, is dependent on external calcium, unaffected by PKC and requires internalisation of the AII-receptor complex (Griendling *et al.* 1993a).

AII then induces the phosphorylation of numerous cellular proteins by calcium-dependent protein kinases, PKC and tyrosine kinases. For instance, AII's action on smooth muscle is thought to be brought about by phosphorylation of the light chain of myosin (Mendelsohn, 1985), while AII exerts a mitogenic effect *via* stimulation of the lipoxygenase pathway to produce 12-HETE, which stimulates cell proliferation; PKC is also involved in this response (Natajaran *et al.* 1992).

It has long been known that calcium plays an important part in AII-stimulated steroidogenesis, and it was thought that this effect was mediated by PKC. Recently, it has been suggested that PKC is less important in the steroidogenic response to AII in bovine adrenocortical cells than was originally thought. Experiments using specific inhibitors to PKC, calmodulin and calcium-calmodulin kinase-II (CAM-kinase II) have shown that Ca^{2+}-calmodulin plays the more important role in stimulation of steroid secretion, and in particular, CAM-kinase II (Ganguly *et al.* 1995). Another recent report demonstrated that AII-mediated steroidogenesis, through the AT_1 receptor, can occur through a different signalling pathway involving inhibition of a novel potassium channel which stimulates calcium entry through depolarisation-dependent channels (Mlinar *et al.* 1995).

1.4.4.2 The AT_2 receptor

Until very recently, the AT_2 receptor subtype was not known to be

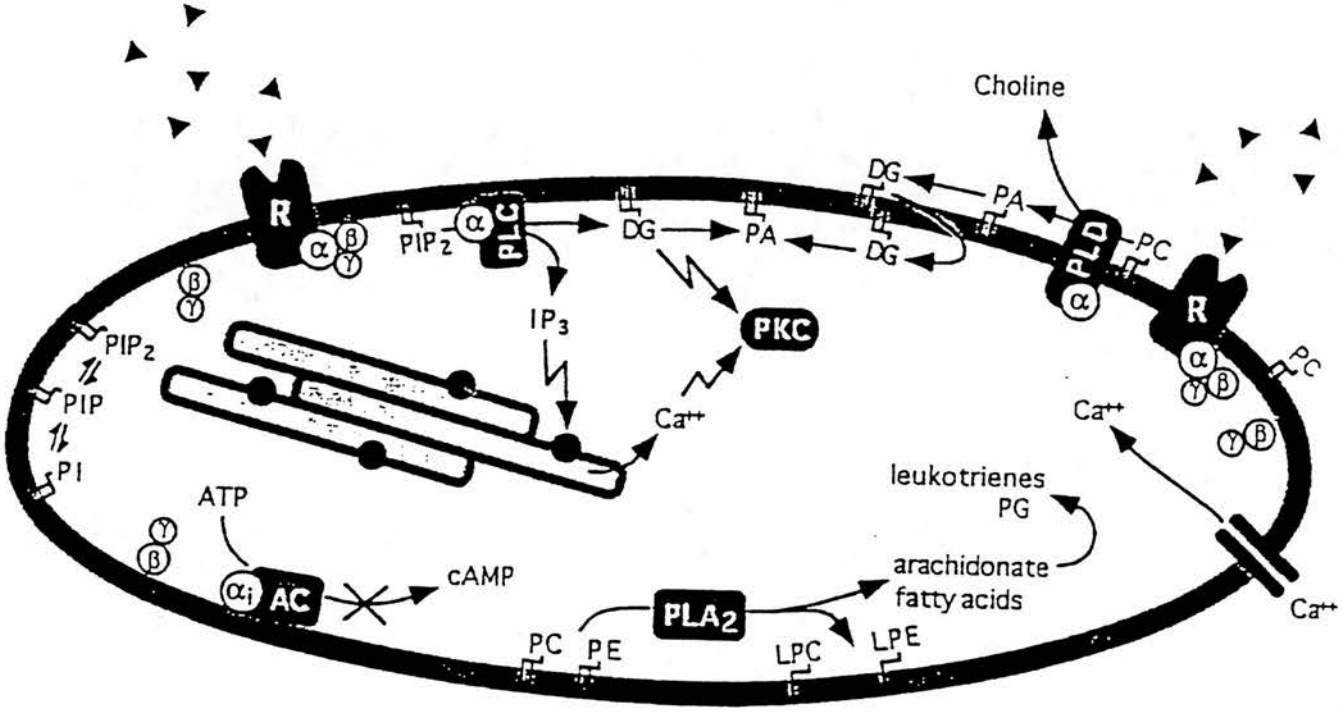


Figure 1.6

Simplified diagram (taken from Griendling *et al.* 1993a) showing signal transduction through the AT_1 receptor, indicating the different systems involved. R = receptor; PLC = phospholipase C; PLA_2 = phospholipase A_2; PKC = protein kinase C; AC = adenylate cyclase; IP_3 = inositol trisphosphate; cAMP = cyclic adenosine monophosphate; PLD = phospholipase D; other abbreviations as in index. Activation of the AT_1 receptor can lead to signalling through IP_3/Ca^{2+}, cAMP, Ca^{2+} influx through T- and L-type channels, leukotrienes and also Jak/STAT (not illustrated; see text).

coupled to any known signal transduction system, nor to mediate any clear physiological response. Early theories postulated that it was a 'silent' receptor or, because it was expressed at a high level in the foetus and neonate, a developmental role was proposed for it (Johnson & Aguilera, 1991; Shanmugam *et al.* 1995). Other reports, after the development of specific antagonists to block any potential functions, suggested a growth-inhibitory role (Nakajima *et al.* 1995; Stoll *et al.* 1995), and a role in modulating cerebral blood flow (Stromberg *et al.* 1992; Naveri *et al.* 1994). There is now evidence that the AT_2 receptor is involved in the regulation of apoptosis (Yamada *et al.* 1996), and indeed its tissue distribution would seem to fit this function well as it is particularly prevalent in tissues in which apoptosis occurs to a large extent (i.e. the zona reticularis of the adrenal cortex).

Following the cloning of the AT_2 receptor subtype (see section 1.3.4) it was proposed that it functions as an inhibitor of protein tyrosine phosphatase (Kambayashi *et al.* 1993b; Kambayashi *et al.* 1994) through a pertussis toxin-sensitive G-protein. Earlier studies, which demonstrated that GTP analogues had no effect on binding of AII or AII analogues to AT_2 receptors, had concluded that this subclass was not coupled to G-proteins (Dudley *et al.* 1990; Aguilera, 1992); the AT_2 receptor in fact belongs to a unique class of G-protein receptors which are not sensitive to GTP analogues, including the dopamine D_3 receptor. Dzau et al (Yamada *et al.* 1996) have suggested that the AT_2 receptor induces dephosphorylation of MAP kinase via activation of a protein tyrosine phosphatase. The relationship of this observation to the demonstration by Kambayashi (Kambayashi *et al.* 1993b) that the AT_2 receptor inhibits protein tyrosine phosphatase is presently unclear. It could be that AII produces a different modulation of protein tyrosine phosphatases in different cell types.

1.4.5 Regulation of AII receptors

Studies on the regulation of AII receptors were initially carried out by examining changes in the number and affinity of AII binding sites in rats after dietary variation (e.g. Aguilera *et al.* 1980) or infusions of AII (Gunther *et al.* 1980). The information gained from these studies was complicated and, in some cases, contradictory, and was only resolved when the distinction between the various AII receptors was understood. The regulation of AII receptors is now appearing increasingly complex, with many local and systemic factors interacting to control the level of expression of the receptors. In this section, the regulation of the two subtypes of AII receptors will be considered separately, discussing what is known about the regulation of the subtype by a particular class of stimulus. Tables 1.2 and 1.3 summarise the regulation of the AT_1 and AT_2 receptors respectively.

1.4.5.1 Regulation of AT_1 receptors

1.4.5.1.1 Variations in ionic balance regulate AT_1 receptor number and affinity

Since sodium levels are important in the control of RAS activity, alterations in sodium status were also thought to be important in the regulation of AII receptors. Aguilera et al investigated such regulation in the rat adrenal zona glomerulosa; 36h sodium restriction doubled AII receptor numbers and increased their affinity; longer restriction increased this effect. However, reciprocal effects were observed when sodium-loaded diets were employed. Parallel changes were also seen in AII-stimulated aldosterone secretion (Aguilera *et al.* 1978).

However, when vascular smooth muscle, the other principal target tissue for AII was examined, opposite effects to those observed in the adrenal zona glomerulosa were observed. Following sodium restriction, AII receptors in mesenteric artery smooth muscle, and non-vascular smooth muscle decreased in number and affinity. The converse was again observed

Stimulus	Human AT_1	Bovine AT_1	Rat AT_1	Rat AT_{1a}	Rat AT_{1b}
Na^+ depletion			↑ (2, 9)	↑ (10)	↑ (10)
Na^+ loading			↓ (2)		
K^+ depletion			↓ (1)		
K^+ loading	↓ (12)		↑ (1, 9)		
AII	↓ (7, 11)	↓ (4)	↑ (2)		↑ (5)
IP_3/Ca^{2+}	↓ (11)	↓ (4)			
cAMP	↓ (11)	↓ (3, 4)			
IGF-1, bFGF		↑ (8)			
ACE inhibitors			↓ (2)		
ACTH		↓ (3)			
insulin		↑ (6)			

Table 1.2

Regulation of the AT_1 receptor in the adrenal gland. This table summarises the information given in the text; where no information is available for a given species, the column is left blank. Only the direction of regulation is shown, as the degree varies with concentration and duration of stimulus The references in parentheses are as follows : 1: Douglas, 1979; 2: Aguilera *et al,* 1980; 3: Andoka *et al,* 1984; 4: Penhoat *et al,* 1988; 5: Inagami *et al,* 1992; 6: Takayanagi *et al,* 1992; 7: Naville *et al,* 1993; 8: Langlois *et al,* 1994; 9: LeHoux *et al,* 1994; 10: Llorens-Cortes *et al,* 1994; 11: Bird *et al,* 1994; 12: Bird *et al,* 1995a; 13: Bird *et al,* 1995c.

following sodium loading (Aguilera *et al.* 1978; Gunther *et al.* 1980; Aguilera & Catt, 1981).

These contradictory results could not be explained by the subdivision of AII receptors into types 1 and 2, as the physiological responses mediated by AII in both adrenal cortex and vascular smooth muscle were due to AT_1 receptor stimulation (Chang & Lotti, 1989). An explanation was given by the cloning of two subtypes of AT_1 receptor (Murphy *et al.* 1991; Iwai *et al.* 1991) in rats, with a reciprocal expression pattern in these two target tissues. The AT_{1a} receptor was preferentially expressed in vascular smooth muscle, while the AT_{1b} predominated in the adrenal cortex. Later studies examining the regulation of these two subtypes confirmed that they were indeed regulated reciprocally by sodium imbalance (Lehoux *et al.* 1994; Sandberg *et al.* 1994; Du *et al.* 1995). Altered sodium status also regulates AT_1 receptor expression in the brain (Ray *et al.* 1990; Sandberg *et al.* 1994).

Imbalanced potassium status is also capable of producing similar reciprocal changes in AT_1 receptors (Douglas, 1979). Potassium loading of rats increases adrenocortical AII receptor number, while decreasing vascular smooth muscle AII receptor number. Again, the converse effect is seen in potassium depletion. Studies have since shown that this is due to reciprocal changes in the two AT_1 receptors (Lehoux *et al.* 1994). In vitro experiments on cultured vsmc have shown similar results (Alexander, 1980; Linas *et al.* 1990).

1.4.5.1.2 AII concentration regulates AT_1 receptors

Variations in sodium levels, as discussed above, can clearly regulate AII receptors. This is due to the alteration produced in the AII levels (Catt & Aguilera, 1980). Experiments where an ACE inhibitor was fed to rats in conjunction with a low sodium diet (Aguilera *et al.* 1980; Gunther *et al.* 1980) prevented the low-sodium-induced changes described above (1.4.5.1.1) from appearing. This indicated that these sodium-induced changes were due to

changes in plasma AII concentration affecting AII receptor number. Infusion of AII into rats (Aguilera *et al.* 1980) mimicked the effects of a low sodium diet in that it produced an increase in AII receptor number in both adrenal zona glomerulosa and renal glomeruli. The reciprocal regulation described above following variations in dietary sodium has also been demonstrated for rat adrenal AT_{1b} and vascular AT_{1a} receptor mRNA (Iwai *et al.* 1991; Iwai & Inagami, 1992b) following AII infusion.

However, such reciprocal regulation of AT_1 receptor numbers in different tissues appears to be restricted to the rat. Treatment of cultured bovine and human adrenocortical cells produces a significant decrease in both AT_1 receptor protein and mRNA (Penhoat *et al.* 1988; Naville *et al.* 1993; Bird *et al.* 1994; Bird *et al.* 1995a). AII also downregulates its receptor protein in many other tissues, including liver (Sernia *et al.* 1985) and human platelets (Moore *et al.* 1984). This would seem to indicate that the upregulation observed in rat AT_{1b} receptor mRNA in adrenocortical and renal tissue in response to AII is an exception.

1.4.5.1.3 AT_1 receptor regulation can be mediated by more than one signal transduction system

The action of AII in regulating its own receptors has been mimicked by the use of phorbol esters and calcium ionophores to stimulate the IP_3/Ca^{2+} signal transduction system. These effects have been demonstrated at both the receptor protein and mRNA levels for rat (Chen *et al.* 1994; Lu *et al.* 1994; Cheng *et al.* 1995), bovine (Penhoat *et al.* 1988) and human (Naville *et al.* 1993; Bird *et al.* 1995a) tissues. Both mRNA and protein levels are regulated in the same direction in response to AII, with the changes in protein levels lagging approximately 6 hours behind those in mRNA levels (Bird *et al.* 1995a).

Activation of the cyclic AMP signal transduction system is also able to regulate AII receptor levels in a number of tissues. In bovine and human

adrenocortical cells in culture, the physiological ligand ACTH produces a significant downregulation of AII receptors (Penhoat *et al.* 1988) and AT_1 mRNA (Andoka *et al.* 1984; Naville *et al.* 1993). This can be duplicated by cAMP analogues and agonists, such as 8-bromo-cyclic AMP and forskolin (Penhoat *et al.* 1988; Bird *et al.* 1995a). Downregulation of AT_1 mRNA by this system has also been demonstrated in rat vsmc and glomerular mesangial cells (Makita *et al.* 1992; Chen *et al.* 1994).

The ability of the AT_1 receptor to be regulated by more than one signal transduction system presents an interesting possibility that receptor and second messenger cross-talk is an important component of AT_1 receptor regulation.

1.4.5.1.4 AT_1 receptors are regulated by steroid hormones

Steroids are potential feedback modulators of the response to AII and, as such, have been examined as influences on AII receptor regulation. Early studies, however, failed to find such a role for any of more than 20 steroids on bovine adrenocortical cells in culture (Campanile & Goodfriend, 1981), although these studies did not involve prior incubation of the cells with steroids, unlike the majority of regulation experiments where prior incubation with the test substance is normal.

Since these experiments, both mineralocorticoids and glucocorticoids have been shown to be capable of regulating AT_1 receptors in tissues other than adrenocortical cells. Aldosterone increases AII binding site numbers in rat vascular smooth muscle cells (Ullian *et al.* 1992), and also upregulates AT_{1a} mRNA levels in the same tissue (Sato *et al.* 1994). The influence of glucocorticoids has been more extensively investigated, with upregulation observed of AII receptor and AT_{1a} mRNA levels in rat vascular smooth muscle cells (Gruenfeld & Eloy, 1987; Sato *et al.* 1994; Ullian & Walsh, 1995). This regulation can be explained by the presence of glucocorticoid response elements in the rat AT_{1a} gene promoter (Guo *et al.* 1995). Glucocorticoids are

also involved in the upregulation of AT_1 receptors in the paraventricular nucleus after exposure of rats to stress paradigms (Aguilera *et al.* 1995).

Vascular AT_{1b} mRNA levels remain unaffected by glucocorticoids (Matsubara *et al.* 1994); however, dexamethasone has recently been shown to downregulate rat adrenal AT_{1b} mRNA expression (Chansel *et al.* 1996). Glucocorticoid-induced downregulation of AT_1 receptor levels has also been shown in pancreatic acinar cells (Chappell *et al.* 1992) and a hepatoma cell line (Wintersgill *et al.* 1995), and cortisol induces downregulation of AT_1 receptors in foetal kidney (Robillard *et al.* 1994; Segar *et al.* 1995). This complex tissue- dependent response to steroid hormones serves to emphasise the complex nature of AT_1 receptor regulation.

1.4.5.1.4 AT_1 receptors can be regulated by growth factors

Growth factors, including IGF-1, PDGF, bFGF and EGF have been reported to increase expression of AT_1 mRNA and receptor protein in cultured rat vsmc (Fujiyama *et al.* 1993; Guo & Inagami, 1994) and bovine adrenocortical cells (Langlois *et al.* 1994). This shows a highly interesting parallel with the growth properties displayed by AII. *In vitro* differentiation of a neuroblastoma cell line, NG108-15, also causes a significant upregulation of AT_1 receptors (Tallant *et al.* 1991).

1.4.5.1.5 AT_1 receptors can be regulated by other physiological changes

Many physiological changes have been shown to affect the levels of AII receptors. For example, high levels of glucose, compatible with those found in diabetics, have been shown to downregulate AII receptor number in rat aortic vsmc (Williams *et al.* 1992); this could be important in the unresponsiveness of the vasculature observed in diabetes.

AT_1 receptor mRNA expression can also be affected by ACE inhibitors independently of their blood-pressure lowering effect, shown by

experiments on the adrenal gland, cardiac myocytes and aortic smooth muscle (Kitami *et al.* 1992; Negoro *et al.* 1994). This regulation is most likely due to the alteration in AII levels induced by these drugs. Myocardial infarction produces an almost two-fold increase in AT_1 receptors (Meggs *et al.* 1993), and experimental renovascular hypertension increases rat AT_{1b} mRNA expression (Llorens-Cortes *et al.* 1994). Adding to the cardiovascular influences which regulate AII receptors, a recent observation (Cahill *et al.* 1995) indicates that enhanced NO generation in rat vsmc leads to a decrease in AII receptor protein. This gives evidence for a possible mechanism of cross-talk between the constricting and dilating factors in the vasculature.

Sex hormones can also regulate AT_1 receptor levels. LH increases, while FSH and testosterone decrease, ovarian AII receptor protein levels (Nielsen *et al.* 1995). Oestrogen also elicits differential regulation of AT_{1a} and AT_{1b} mRNA levels in the pituitary, only the latter showing an increase in response to this hormone (Kakar *et al.* 1992).

AT_1 receptor regulation thus involves the interplay of many stimuli and, in rodents, differential regulation of the two AT_1 gene products.

1.4.5.2 Regulation of AT_2 receptors

Much less is known about the regulation of the AT_2 receptor in comparison with the AT_1 subtype. In keeping with the proposed growth-modulatory role of this receptor (Shanmugam *et al.* 1995; Nahmias & Srosberg, 1995; Nakajima *et al.* 1995), AT_2 receptor mRNA levels vary in a growth-dependent manner (Kijima *et al.* 1995), being inversely related to the mitogenic activity of the cells in which it is expressed (Inagami *et al.* 1995). Peptide growth factors (PDGF and EGF) induce downregulation of AT_2 receptors in rat aortic smooth muscle cells (Kambayashi *et al.* 1993a; Kijima *et al.* 1995), while stimulating growth of the cells and increasing levels of AT_1 receptor. Insulin, which can also act as a growth factor, has also been shown to upregulate AT_2 mRNA (Ichiki *et al.* 1995). AT_2 receptor numbers also

increase in wound healing (Viswanathan & Saavedra, 1992) and in injured tissues after balloon catheterisation (Pratt *et al.* 1992), although, conversely, Viswanathan *et al.* (1992) showed only an increase in AT_1 receptors. The AT_2 receptor has been reported to be involved in apoptosis (Yamada *et al.* 1996), and the AT_2 receptor was also found to be downregulated in R3T3 cells upon reaching confluence (Ichiki *et al.* 1995). The gene is also developmentally regulated, as evidenced by the fact that its expression is decreased or abolished in many tissues shortly after birth (Ichiki & Inagami, 1995). Pregnancy is associated with downregulation of uterine AII receptors (Yang *et al.* 1994). A recent study in pregnant women by de Gasparo et al showed that this was due to a decrease in expression of the AT_2 receptor (De Gasparo *et al.* 1994); the small number of AT_1 receptors are not affected and so the proportion of AT_1:AT_2 is increased. Both AT_1 and AT_2 receptors are upregulated by differentiation of the NG108-15 cell line (Tallant *et al.* 1991), but the latter to a greater extent, suggesting their involvement in the differentiation process. When N1E-115 cells were treated with AII in a study by Reagan *et al.* (1993), a downregulation of AII receptors was observed; the AT_2 receptors were downregulated to a greater extent than the AT_1 receptors, although the latter were downregulated more quickly.

When the existence of two AII receptor subtypes was initially proposed, it was thought that they would be oppositely regulated. In other words, an increase in AT_1 would be accompanied by a decrease in AT_2 and *vice versa*. This has not, so far, proved to be the case and it will be interesting to see what results future experiments on the mechanisms of AT_2 receptor regulation will yield.

1.4.5.3 Mechanisms and significance of AII receptor regulation

AII receptors can be regulated in a tissue, species, age and subtype-specific manner, and at different stages in their production (translation, transcription etc).

Stimulus	Human AT_2	Rat AT_2	Mouse AT_2
PDGF, EGF		↓ (rat vsmc; 3, 6)	
insulin			↑ (R3T3 cells; 5)
wound healing	↑ (dermis, 2)		
confluence			↑ (R3T3 cells; 5)
pregnancy	↓ (myometrium; 4)		
differentiation			↑ (N1E-115 cells; 1)

Table 1.3

Regulation of the AT_2 receptor. Again only direction of regulation, and not degree, is shown. References in parentheses are listed below: 1: Reagan *et al,* 1990; 2: Viswanathan *et al,* 1992; 3: Kambayashi *et al,* 1993a; 4: de Gasparo *et al,* 1994; 5: Ichiki *et al,* 1995; 6: Kijima *et al,* 1995.

Regulation of a receptor can be achieved at three levels. The first stage is to control the activity of the receptor by altering its phosphorylation; this contributes to observed changes in affinity. The second is by internalisation of the receptor, and this is apparent in the initial stages of all downregulation. These are short-term measures for reactions to acute stimuli; included in these are changes in mRNA production and stability. For chronic regulation, more wide-reaching effects are necessary; these include *de novo* synthesis of new receptors. This comprises the third stage of receptor regulation. Depending on the nature and length of stimulus applied, some or all of these stages may be affected. For example, in the regulation of AT_1 receptors in the human adrenocortical cell line H295, AII stimulation results in a rapid downregulation of the mRNA and protein, with recovery occurring after 24-48h stimulation (Bird *et al.* 1994). The initial downregulation is achieved through internalisation of the receptor and decreasing of message stability, while the recovery phase is dependent on *de novo* receptor protein synthesis (Bird *et al.* 1995a), indicating the involvement of both transcriptional and translational control. A similar complexity of mechanism is seen in the downregulation of AT_1 receptors in rat vsmc by AII (Lassegue *et al.* 1995). Different regulatory agents may affect these stages to varying extents, as shown by Makita *et al.* (1992), where the downregulation induced by AII was shown to differ from that induced by cAMP by the fact that blockade of *de novo* protein synthesis by cycloheximide abolished the downregulatory effect of AII but not cAMP.

The differential regulation of AT_1 receptor subtypes in different tissues indicates distinct functional specificities (Du *et al.* 1995) and could serve as a mechanism by which AII-dependent functions are locally regulated (Aguilera & Catt, 1981). Little is known about the mechanisms of AT_2 receptor regulation, and it is difficult to speculate on its significance, although if it is involved in mediating apoptosis then abnormalities in its expression and function could have wide-reaching implications.

The homologous and heterologous downregulation of AT_1 receptors at both the protein and mRNA levels, by any or all of the mechanisms mentioned, may participate in the co-ordinated physiological adaptation of vascular tone and electrolyte balance (Lassegue *et al.* 1995). Alterations in AII receptor expression may provide the pathological basis for abnormalities in blood pressure, electrolyte and fluid volume homeostasis (Du *et al.* 1995); the complex nature of AII receptor regulation makes it apparent that a clear understanding of how this is achieved is necessary if we are to pharmacologically modify it in any way.

1.5 Use of animal models for studying the action of AII

In this section I will discuss the animal cell culture models I have used predominantly in this thesis, and justify the choice of these models in comparison to the others available. This will encompass primary cultures only; a consideration of the role of tumour-derived cell lines can be found in Chapter 6.

1.5.1 The bovine adrenal cortex

During this thesis I have used both zona glomerulosa and zona fasciculata/reticularis cells in primary culture. The zona glomerulosa has been the most commonly used model for studying AII action, whether looking at receptor binding, physiological response or mRNA expression, as it is the main site of AII action *in vivo* in the adrenal gland. Although many investigators have used primary cultures of bovine zona glomerulosa cells, as well as bovine zona fasciculata/reticularis cells, an equally large number have investigated the rat zona glomerulosa. This is principally because physiological and dietary investigations can be carried out in the rat, whereas this is not easily possible in cattle.

The rodent adrenal model, however, has a number of limitations. Rat zona glomerulosa cells do not divide in culture, unlike bovine cells, which can lead to problems for longer-term *in vitro* studies. There is also a major difference in the steroids produced in adrenal cortexes of the two species, and it is the bovine adrenal cortex, which produces cortisol as the major glucocorticoid, which parallels the human situation. The major glucocorticoid produced in the rat adrenal cortex is corticosterone, this difference making it a less suitable model for human steroidogenesis.

Another reason for working with the bovine adrenal cortex is that the bovine zona fasciculata, like the human zona fasciculata, responds to AII by secreting cortisol (McKenna *et al.* 1978; Clyne *et al.* 1993). The rat zf, however, is unresponsive to AII. Additionally, analysis of the AT_1 receptor, discussed in 1.4.4.1, indicates that the non-divided nature of the bovine AT_1 receptor is more similar to the human than is the rat. The bovine model has one final advantage over the rat : the ease of obtaining the tissue. It is for a combination of these reasons that the bovine adrenal cortex was used as a model for this thesis.

1.5.2 The rat mesenteric artery (vascular smooth muscle)

Whereas the bovine species is more similar to man where steroidogenesis is concerned, the rat is generally accepted to be a close model for the human cardiovascular system. Again, physiological manipulations are easier to perform, and rat aortic and mesenteric artery smooth muscle preparations have been frequently used for studies of AII binding and actions, especially *in vitro* (Aguilera & Catt, 1981; Lyall *et al.* 1992). Bovine vascular smooth muscle has been employed in studies of AII action (Gruetter *et al.* 1988; Murray *et al.* 1990); however, this has been mostly large vessel smooth muscle cells and as discussed earlier (1.3.2), the elastic arteries are not the most responsive to AII. The rat model would therefore seem to be the better one, as more data is available for comparison.

However, the different number of AT_1 receptor genes in the rat and human must be considered.

1.6 Aims of the thesis

1. To establish a modified primary culture system for bovine adrenocortical zona glomerulosa cells, quicker and easier than established methods.

2. To resolve previously observed discrepancies (suggesting differences between the receptors) in the pharmacological characteristics of the type 1 angiotensin II receptor in bovine adrenal zona glomerulosa and rat vascular smooth muscle by the use of Schild analysis to obtain pA_2 values.

3. To PCR-amplify, using primers designed against the published sequence, the bovine adrenal AT_1 receptor coding region and to generate and sequence a clone of this region, and from the clone to purify a DNA sequence suitable for use as a probe in Northern blots.

4. To use semi-quantitative Northern blot analysis to study the regulation, by AII, signal transduction systems and steroids, of AT_1 receptor mRNA expression in primary cultures of bovine zona fasciculata/reticularis cells. Northern analysis of AT_1 mRNA was chosen rather than study of receptor protein by ligand binding for two main reasons : to avoid possible distortion of ligand binding results through internalisation of receptors after exposure to AII, and to obtain information on the transcriptional regulation of AT_1

receptor mRNA levels, to complement what is already known about the regulation of the AT_1 receptor protein.

Chapter 2 : Materials and Methods

2.1 Materials

All chemicals were AnalaR grade and except where otherwise stated were obtained from BDH Merck or Sigma Chemical Co.

Aldrich Chemical Co. Ltd., Gillingham, Dorset

1,1,2-trichlorotrifluoroethane, toluene, tri-n-octylamine.

Amersham International PLC, Little Chalfont, Buckinghamshire

[α-^{35}S]dATP, [α-^{32}P]-dCTP, cortisol-3-CMO-2(^{125}I)-iodohistamine, myo-^{3}H-inositol, Na^{125}I, Sequenase kit (from USB).

Amicon Ltd., Stonehouse, Gloucestershire

Microconcentrator columns, Micropure inserts.

Applied Biosystems, Warrington

ULTma DNA polymerase.

BDH Merck Ltd., Lutterworth, Leicestershire

Absolute ethanol, acrylamide solution (40%, stabilised), chloroform, dimethylformamide, dimethylsulphoxide, ethyl acetate, Ficoll, formamide, formic acid, glass Allihn funnels, glycerol, isopropanol, lithium chloride, PEG, perchloric acid, polyvinylpyrrolidone, Triton X-100, urea.

Bio-rad Laboratories Ltd., Hemel Hempstead, Hertfordshire

AG1X8 anion exchange resin, β-mercaptoethanol.

Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Lewes, E. Sussex

Adenosine triphosphate, agarose, caesium chloride, deoxynucleotides, EDTA, Klenow enzyme, λ phage DNA, positively charged nylon membrane, proteinase K, restriction enzymes, trypsin.

Ciba Laboratories, Horsham, W.Sussex

Synacthen 250μg (ampoule; ACTH 1-24).

Fisons Scientific Equipment, Loughborough, Leicestershire

Phio-Bio Tris-equilibrated phenol.

Gibco BRL (Life Technologies) Ltd., Paisley

Amphotericin B, Dulbecco's modified Eagle medium (DMEM), Earle's balanced salts (EBS), Ham's F-10, inositol-free DMEM, nuclease-free bovine serum albumin (BSA), penicillin/streptomycin, Seachem agarose, T4 DNA ligase, T4 DNA polynucleotide kinase.

Greiner Labortechnik Ltd., Stonehouse, Gloucestershire

Sterile 30mL and 50mL plastic containers.

Hayman Ltd., Witham, Essex

Absolute ethanol.

ICN Flow (ICN Biomedicals) Ltd., Thame, Oxfordshire

Linbro tissue culture-treated plastics (6, 12, 24, 96-well plates), soybean trypsin inhibitor.

Lockertex, Warrington

Nylon gauzes (20, 30, 100, 250μm pore).

Mackay & Lynn Ltd., Edinburgh

Bibby Sterilin sterile 30mL plastic containers, Corning tissue culture plasticware (25, 75cm^2 flasks), Neubauer improved haemocytometer, Whatman 3MM chromatography paper.

New England Biolabs (UK) Ltd., Hitchin, Hertfordshire

Restriction enzymes (BamHI, BSpMI, DraI, EcoRI, HinDIII, MunI, SalI, SSpI).

New England Nuclear (DuPont NEN), Stevenage, Hertfordshire

RIAfluor scintillation fluid.

NIBSC, Potters Bar, Hertfordshire

Angiotensin II (asp^1-val^5; MRC standard 64/15), insulin-like growth factor 1 (International reference standard 87/518).

Oswel DNA Service, Southampton, Hampshire

All PCR, random and sequencing primers.

Parke-Davis, Pontypool, Gwent

Arginine vasopressin (as pitressin).

Pharmacia Biotech Ltd., Milton Keynes

DEAE Sephadex A25, dextran, NAP 5 & 25 columns, Percoll.

Promega Corporation, Southampton, Hampshire

Taq DNA polymerase.

Qiagen Ltd., Dorking, Surrey

Qiaex kit.

Sanofi Winthrop Ltd., Guildford, Surrey

Noradrenaline (tartrate; as levophed).

Scottish Antibody Production Unit (SAPU), Carluke, Lanarkshire

Antisera to cortisol, non-immune sheep serum.

Sigma Chemical Co. Ltd., Poole, Dorset

8-bromo-cyclic AMP, A23187 (calcium ionophore), agarose, aldosterone, ammonium formate, ammonium persulphate, ampicillin, bromophenol blue, BSA (cell culture grade), charcoal, Chloramine T, cortisol, CPSR-1, D-glucose, elastase (porcine), ethidium bromide, FCS, glyoxal, guanidinium thiocyanate, histamine, Hoechst 33258 dye, horse serum, hydrogen peroxide (30% v/v), inositol, isobutylchloroformate, light mineral oil, lysozyme, methylene blue, n-lauroyl sarcosinate (sarcosyl), phorbol myristate acetate (PMA), RNAse A, salmon sperm DNA, saralasin, Sephadex G-10, G-25 and G-50 gels, sodium lauryl sulphate, sodium metabisulphite, TEMED, tributylamine.

Steraloids UK, Ltd.

Aldosterone 3-methyl mono-oxime.

Worthington : Lorne Laboratories Ltd., Twyford, Berkshire

Collagenase (type 1).

The following were obtained from non-commercial sources. The angiotensin II receptor antagonists DuP753 and PD123177 were obtained from the DuPont Experimental Station, Wilmington, Delaware, USA. *E.Coli* cells strain DH5, the cloning vector pSP72poly4, and a 1.25kb cDNA fragment containing the protein coding region of rat β-actin were kind gifts from Dr. S. Morley, BBSRC Centre for Genome Research, King's Buildings, University of Edinburgh.

2.2 Methods

2.2.1 Cell culture methods

2.2.1.1 Bovine adrenal cortex zona glomerulosa

Bovine adrenal glands were obtained (from freshly slaughtered 1.5 - 2-year-old steers) on ice from the local slaughterhouse and trimmed of fat. At this stage the glands were dipped in 70% ethanol, transferred to a class 2 sterile cabinet and aseptic technique was employed. Following fine trimming of adherent fat, 100μm slices were cut from the glands using a Stadie-Riggs microtome. Outer slices, comprising the capsule and the zona glomerulosa, with the outermost part of the zona fasciculata (although this was not obviously visibly present), were placed in a 50mL plastic container with ~15mL EBS/0.2% BSA until about 10g had been collected. The slices were then chopped very finely with sharp scissors (1mm fragments) and washed with EBS/0.2% BSA before digestion with 50mL collagenase type I (2mg/mL in EBS/2% BSA) for 40min at 37°C. The tissue was dispersed after 20min by pipetting up and down using a 5mL pipette with a wide bore, and again after 40min using a finer tip. The cell suspension was then filtered through 250 and 100μm mesh nylon gauzes before being centrifuged (30min, 450g) to pellet the cells. The supernatant was discarded and the pelleted cells resuspended in EBS/0.2% BSA and passed through a 30μm gauze to yield a suspension of single cells. This was further purified by one of two methods, either the Percoll density gradient method as in Shepherd *et al.* (1992), or a modification of the gel filtration method described in 2.2.1.2.

The first method involved layering the cells (in a volume of approximately 2mL) onto a discontinuous Percoll density gradient (75,40,30 & 20% Percoll in EBS/0.2% BSA) and centrifuging for 20min at 800g. The zona glomerulosa cells were found at the 30-40% Percoll interface and removed using a glass Pasteur pipette. They were washed twice with

EBS/0.2% BSA and spun (450g, 10min) to remove contaminating traces of Percoll. The cells were then filtered again through a 20μm mesh nylon gauze, made up to a volume of 20mL in growth medium (Ham's F-10 supplemented with 10% CPSR-1, 100IU/mL penicillin, 100μg/mL streptomycin and 25μg/mL amphotericin B) and counted using a haemocytometer. Cells were plated out into 96-well microtitre plates at a density of 20,000 cells/well (in 200μL growth medium/well); yield was typically $10X10^6$ cells. The cells were maintained in an incubator at 37°C with a humidified atmosphere of 95% air: 5% CO_2; medium was replaced every second day.

The second method used for purification of the zg cells was a modification of the Sephadex column used in 2.2.1.2. The cell suspension was passed through a similar column, made up of Sephadex G-10 on top of G-25 (superfine); cellular debris passed through the column on gentle suction and the cells were retained. The cells were freed by resuspending the gel in about 50mL EBS/0.2% BSA and filtering through a 20μm mesh nylon gauze, which retains the Sephadex gel but permits the cells to pass through. The cells were pelleted by centrifuging (30min, 450g), resuspended and counted as previously. Yield was similar to the Percoll method and the cells were maintained identically, before being used for experiments as described in 2.2.2.1.

2.2.1.2 Bovine adrenal cortex zona fasciculata/reticularis

Bovine adrenal glands were obtained and trimmed as described in 2.2.1.1. 100μm slices were then taken with a Stadie-Riggs microtome as before. The outer slices were discarded; successive inner slices were placed into a 50mL plastic container containing EBS/0.2% (w/v) BSA. Slices appearing to contain medulla (identified by a distinct colour change) were discarded. When about 5g of tissue had been collected, the slices were chopped using sharp scissors into 1-2mm fragments, washed in

EBS/0.2% BSA and digested for 2 hours in a 50mL solution of collagenase type I (2mg/mL in 2% BSA) at 37°C, with frequent shaking (30 minute intervals) to disperse the cells. The resultant cell suspension was then filtered through successive 250 and 100μm mesh nylon gauzes to remove undigested tissue and then centrifuged (20min, 450g) to pellet the cells. After discarding the supernatant, the cells were resuspended in EBS/0.2% BSA and filtered through a 30μm gauze to give a single cell suspension, which was then purified using the column filtration method of McDougall *et al.* (1979). The cells were drawn, using gentle suction, through a column consisting of a sintered glass Allihn funnel (100mm x 20mm disc; 16-40μM pore size) containing 5mL Sephadex G-50 (fine) layered on 15mL Sephadex G-10 (fine). This removed red blood cells and other cell debris which flowed through the column, leaving the intact cells trapped in the Sephadex gel. The cells were removed from the gel by resuspending it in approximately 50mL of EBS/0.2% BSA and filtering this through another 30μm pore nylon gauze to trap the gel beads while allowing the cells to pass through. The resulting suspension was then centrifuged (30min, 450g) to pellet the cells. The pelleted cells were resuspended in 50mL growth medium (identical to that used for the zg cells; 2.2.1.1). Yield was determined by use of a haemocytometer and was normally 60-80X10^6 cells. The cells were then plated out in either 12-well plates at a density of 4X10^6 cells /plate (1mL/well), or in 25cm^2 cell culture flasks at a density of 5X10^6 cells/flask (5mL/flask). Medium was changed daily, serum being withdrawn when required when experiments commenced on day 2 in culture (day 0 = day of isolation). The cells were maintained as described for the zg cells in 2.2.1.1, and used as described in section 2.2.2.9 for experiments.

(following the method of Williams et al (1989))

2.2.1.3 Bovine pulmonary artery smooth muscle *

Sections of bovine pulmonary artery (approximately one inch long, as near to the heart as possible) were obtained on ice from the local

* method adapted from Freshney, 1987.

slaughterhouse. Tissue was obtained within 30 minutes of slaughter, from 1-2-year-old steers, and was transported to the laboratory in EBS containing antibiotics (penicillin/streptomycin as described below)

Under sterile conditions, the section was cut into an oblong strip and placed endothelium-side up on a glass plate. The endothelium was scraped off using a sterile scalpel and the artery scored in 1mm-apart lines. Strips of tissue were then cut away from the artery, comprising the innermost two-thirds of the tissue. These strips were cut into 1-2mm lengths and placed inner side down in 6-well plates. 5-6 of these tissue explants were put in each well. The explants were left uncovered for 30 minutes to ensure attachment to the plate. 1mL of growth medium was then put into the wells (growth medium : DMEM + 10% FCS + 200IU/mL penicillin, 200μg/mL streptomycin and 50μg/mL amphotericin B). The plates were then covered and placed in an incubator, and cultured at 37°C in a humidified atmosphere of 5% CO_2, 95% air. The medium was replaced every two days; after two weeks the concentration of antibiotics in the medium was halved. About two weeks after initial isolation, cells started to grow out of the explants and when sufficient cells had appeared, the explants were removed using sterile forceps. When the cells reached confluence, they were passaged.

For passaging, the medium was discarded and the cells washed with 1ml Versene (0.05% trypsin, 0.2% EDTA, 0.085% NaCl in PBS). This Versene was removed and a further 1ml added. This was left to incubate at 37°C for 2-3 minutes until the cells had detached from the bottom of the flask. A little growth medium was then added to the flask and the mixture centrifuged (20min, 450g). The pellet was resuspended in an appropriate volume of growth medium and the cells split in a ratio of 1:3 to 1:5 with respect to surface area. For experiments the cells were passaged as described (using 3ml Versene for a 75cm^2 flask) and counted before being plated into 24-well plates at a density of 50,000 cells/well (0.5mL/well). The cells were then allowed to grow to confluence before being used, routinely between

passages 4 and 7. Identity of the cells was confirmed with immunohistochemical staining for smooth muscle actin and myosin.

2.2.1.4 Rat mesenteric artery smooth muscle

Mesenteries were excised by blunt dissection from 6 male Sprague-Dawley rats (150-200g) and placed in sterile dishes containing DMEM with 100IU/ml penicillin, 100μg/ml streptomycin and 25μg/ml amphotericin B. The dishes were then transferred to a class 2 sterile cabinet and the rest of the procedure (according to Lyall et al, 1992) was carried out under sterile conditions. Fat was cleaned off the arteries using fine forceps and the small arterioles were trimmed off. The trimmed mesenteries were then transferred to 10ml of an enzyme solution (1.25mg/ml collagenase type I, 0.25mg/ml elastase and 0.05mg/ml soybean trypsin inhibitor in DMEM supplemented with antibiotics as before) for 10min at 37° C. The arteries were then removed from the enzyme mix and any remaining adventitia and fat were removed by fine trimming; the endothelium was also removed at this stage by rubbing the arteries along their length between the arms of a pair of fine forceps. The arteries were then cut into 1-2mm pieces and transferred into 10ml fresh enzyme solution and incubated at 37°C until a single cell suspension was achieved. This normally took about 20min, with the suspension being passed up and down through a sterile Pasteur pipette every 10 min. The cells were then centrifuged (20min, 450g), the supernatant discarded and the pellet resuspended in 10mL growth medium (DMEM containing 10% FCS, 10% horse serum, 100IU/ml penicillin, 100μg/ml streptomycin and 25μg/ml amphotericin B) and plated out into a 25cm^2 flask. The cells were grown at 37°C in a humidified atmosphere of 95% air : 5% CO_2. Medium was changed after 48hrs and the cells grown to confluence.

When the cells reached confluence, they were passaged in the manner described in 2.2.1.3, in a ratio of 1:4. Cells were routinely used between passages 4 and 7; for experiments they were plated at the same density as the

bovine vsmc. Identity of the cells as vascular smooth muscle cells was confirmed by staining for smooth muscle actin, myosin and desmin.

2.2.2 Biochemical methods

2.2.2.1 Stimulation of steroidogenesis in cultured cells

Bovine adrenal zona glomerulosa cells were plated into 96-well plates at a density of 20,000 cells/well and cultured for 5 days (including day of isolation). The medium was then removed and the cells washed three times with 200μL/well EBS/0.2% BSA/0.1% glucose. After washing, 120μL growth medium (as in 2.2.1.2) was put into each well and antagonist, if required, or growth medium for controls, was added in a volume of 15μL and mixed in by swirling. The plates were returned to the incubator for 5 minutes to allow antagonist to bind to the receptor and then the agonist was added and mixed, also in a volume of 15μL. The plates were then incubated at 37°C for three hours and the medium collected into 0.5mL tubes; these were stored at -20°C prior to measurement of steroid secretion by RIA. Cells were taken from three wells per plate for protein analysis (see 2.2.2.4).

2.2.2.2 Radioimmunoassay for aldosterone

Aldosterone secretion from zona glomerulosa cells was measured using an "in-house" radioimmunoassay optimised in the Department of Medicine, Western General Hospital, Edinburgh. (adapted from DeMan et al, 1980)

Standards representing 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 & 51.2nM aldosterone were prepared in Ham's F-10 nutrient medium, as well as a standard of 640nM to estimate non-specific binding (NSB). Samples of 0.5. 5 and 20nM were used as QC (Quality Control) samples to estimate interassay variation and intra-assay drift (found to be both less than 10%). Standards, QC and

NSB samples were frozen in aliquots of 150μL and used only once. NSB was typically less than 5%.

Aldosterone was iodinated with ^{125}I as described in 2.2.2.7 and used in the assay at 4000cpm/100μL. This tracer and the antibody (used routinely at a titre of 1:150 dilution from 1:100 stock) were diluted in 0.1M phosphate buffer, pH 7.4, containing 0.1% (w/v) sodium azide and 0.1% (w/v) BSA. 25μL standard or sample was mixed with 100μL antibody and 100μL aldosterone tracer in LP4 assay tubes and incubated overnight at 4°C. Separation of bound from free tracer was effected by the addition of 500μL cold (4°C) charcoal suspension (0.6% (w/v) activated charcoal, 0.06% (w/v) dextran T70, 0.04% gelatine in 0.1M phosphate buffer pH7.4), vortexing and centrifugation (450g, 20min, 4°C). The supernatant, containing antibody-bound aldosterone, was aspirated and discarded and the free iodinated aldosterone, bound to charcoal, remaining in the pellets was counted on a Nuclear Enterprises gamma counter (NE1600) for 3 minutes. A specimen standard curve for this assay is shown in Figure 2.1. Curves were fitted to a 4-parameter logistic model by the gamma counter.

2.2.2.3 Radioimmunoassay for cortisol

An 'in-house' method developed by staff at the Department of Medicine, Western General Hospital, Edinburgh*, was used to determine secretion of cortisol from cultured adrenocortical cells. The assay used a polyclonal antibody to bind to the cortisol and a second polyclonal antibody to separate the free and bound phases.

Standards representing 0, 1, 2, 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120 nM cortisol were prepared in EBS/0.2% BSA/0.1% glucose and frozen in 50μL aliquots. Quality control samples representing 8, 80 and 800 nM were also prepared. Inter- and intra-assay variations were routinely found to be <10%.

* Modification by Williams (unpublished) of the method of Gray + Seth, 1983.

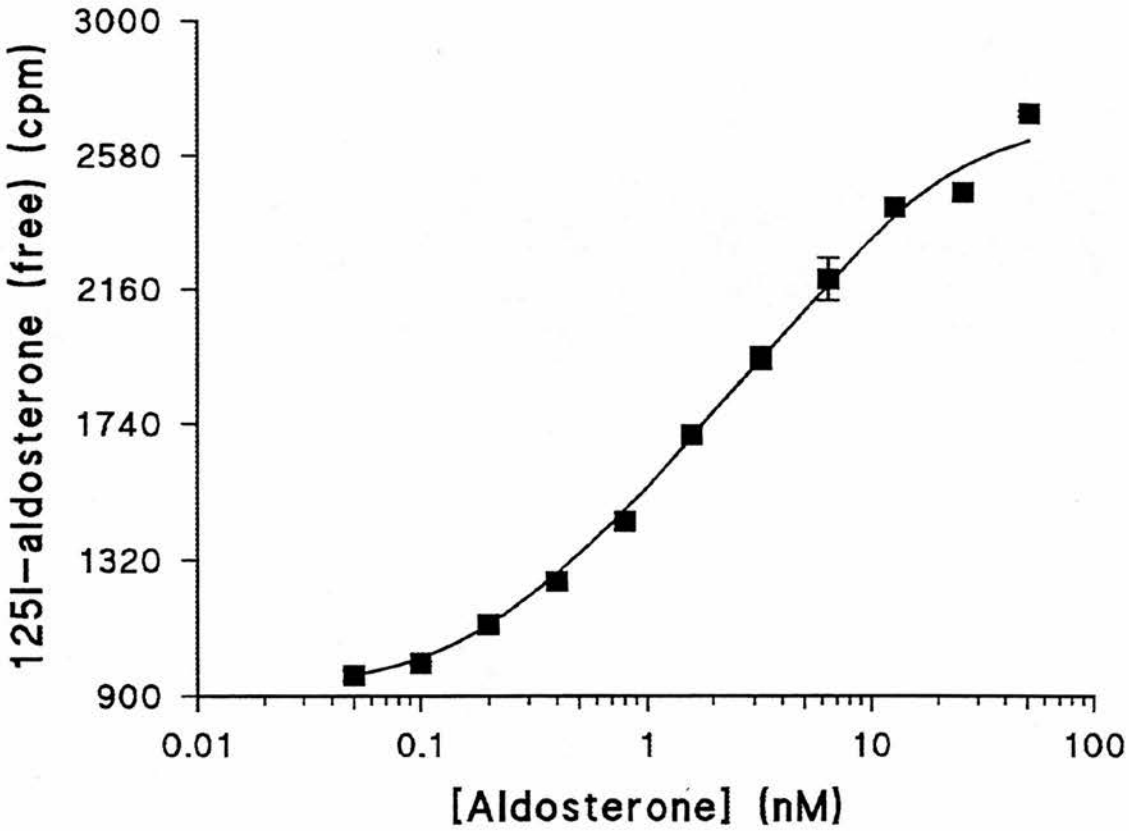


Figure 2.1

Typical standard curve for the aldosterone RIA described in 2.2.2.2. Standards in EBS/0.2% BSA, 0.02-51.2nM were assayed in duplicate and the curve fitted to a four parameter logistic model. Label binding in the absence of aldosterone was typically 60-70% of total.

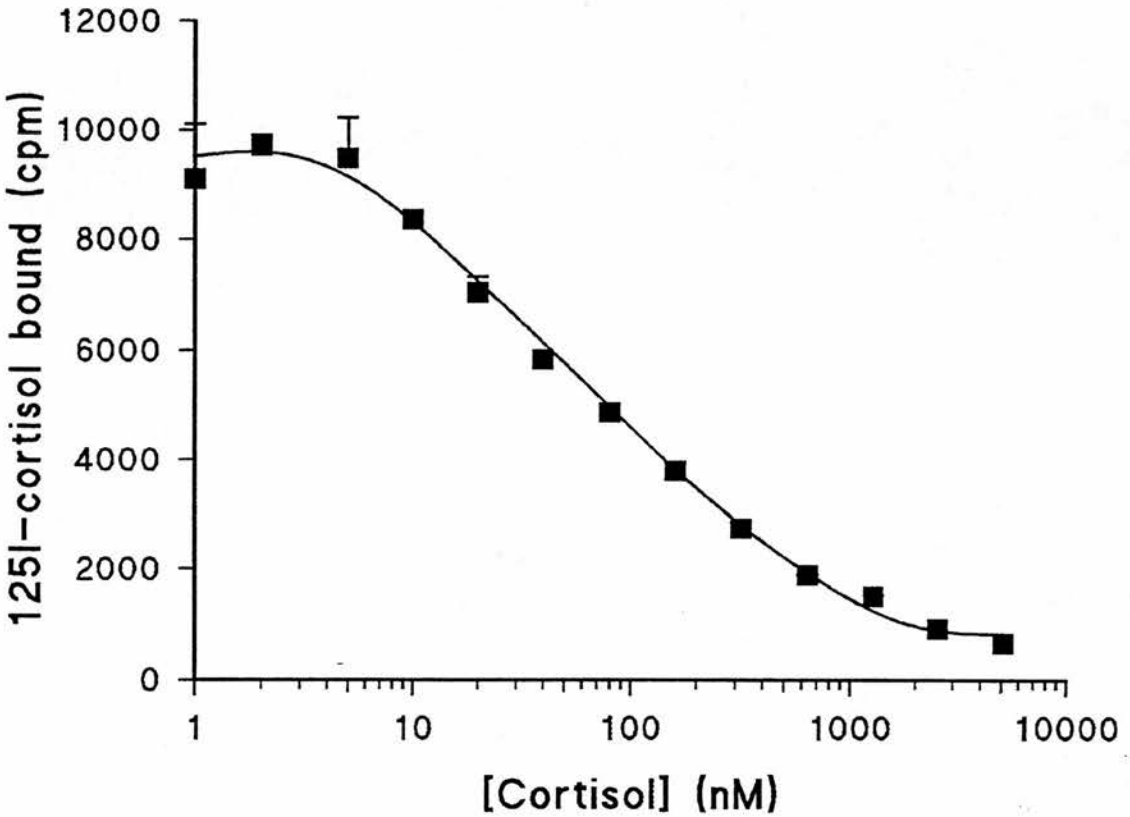


Figure 2.2

Typical standard curve for the cortisol RIA described in 2.2.2.3. Standards in EBS/0.2% BSA, 1-5120nM, were assayed in duplicate and the standard curve fitted to a four-parameter logistic model. Label binding in the absence of cortisol was typically 60-70% of total.

10μL of sample/standard were pipetted into polypropylene tubes. To these were added 50μL ^{125}I-cortisol (cortisol-3-CMO-(2-(^{125}I)iodohistamine)) (diluted in assay buffer : 100mM citrate-phosphate pH 4.0 containing 0.02% (w/v) gelatine and 0.0013% (w/v) sodium azide; to yield ~10,000cpm/50μL) and 50μL primary antibody (used at a dilution of 1:800; final dilution = 1:3200).

Two tubes containing label alone were also prepared. The tubes were then well mixed and incubated for 4 hours at room temperature. 50μL each of the second antibody (initial dilution of 1:15 in assay buffer) and non-immune sheep serum (initial dilution of 1:200 in assay buffer) were then added to the tubes, mixed well and incubated overnight at 4°C. 2mL of cold assay buffer were then added, the tubes vortexed and then centrifuged for 45 minutes at 3,000rpm at 4°C. The supernatant was then tipped off, the tubes blotted dry on some cellulose wadding and then counted for 120 seconds in a gamma-counter (LKB 1621 Multigamma). A specimen standard curve is shown in Figure 2.2. The data were analysed using RIA-calc software, the curve being fitted to a 4-parameter logistic model.

2.2.2.4 Protein estimation

Protein was analysed by the method of Bradford (1976) using a method adapted for a Cobas Fara centrifugal analyser. This was used to normalise responses (e.g. steroid secretion) to the protein content in a particular well. Briefly, at the end of an experiment cells were rinsed in 0.9% saline and then solubilised and detached from the plate by addition of 0.5mL 1% Triton X-100 and gentle scraping. Samples were then diluted 1:10 and analysed against standards of BSA from 0-100μg/ml in 0.1% Triton X-100.

2.2.2.5 ^{125}I-AII binding experiments with cultured cells

Bovine adrenal zona glomerulosa cells were added to 24-well culture plates at a density of 200,000 cells/well in 1mL growth medium. The cells were cultured for 5 days including the day of isolation. Medium was changed on the first and third days. The cells were then washed three times with EBS/0.1% BSA, 1mL/well. After this, 0.8mL EBS/0.1% BSA was added to each well and the plates were incubated at room temperature for 20 minutes.

0.1mL of either EBS/0.1% BSA, agonist/antagonist or 10μM AII (for non-specific binding) was then added. The plates were then swirled to mix the solutions and the ^{125}I-AII was added in a volume of 0.1mL; 100,000cpm/well (typically 0.05pmoles). The plates were again swirled and incubated at room temperature for 150 minutes to allow equilibrium to be reached.

At the end of the incubation period, the EBS/0.1% BSA/AII solution was removed from the wells and discarded. The cells were rapidly washed with 3X1mL ice-cold PBS, the rinses also being discarded. 1mL 1% Triton X-100 was then added to each well and the cells removed by gentle scraping with the plunger of a 2mL plastic syringe. The cell suspension was transferred to LP4 polypropylene tubes and counted for 60 seconds in a Nuclear Enterprises gamma counter (NE1600). Non-specific binding was typically less than 5%.

2.2.2.6 Measurement of phosphatidylinositol turnover

Stimulation of phosphatidylinositol turnover by AII and other agonists was measured in vascular smooth muscle cells as a means of quantifying the magnitude of agonist stimulation in the absence of being able to measure an end-organ contractile response. Methods developed 'in-house' by I.M. Bird. (Bird et al, 1992; Clyre et al 1992)

2.2.2.6.1 Labelling of cells

Rat or bovine vascular smooth muscle cells were plated into 24-well plates at a density of 50,000 cells/well (0.5mL/well) in normal growth medium and incubated for 24 hours at 37°C. The medium was then removed and replaced with 0.5mL serum-free medium (see 2.2.1.3) prepared using inositol-free DMEM containing ^{3}H-inositol to a concentration of 10μCi/mL. The plates were then replaced in the incubator and left for 72 hours to allow steady-state turnover of labelled phospholipids to be reached. The cells were then ready for experiments.

2.2.2.6.2 Stimulation of phosphatidylinositol turnover

The labelled medium was removed from the cells after 72 hours and replaced with 0.5 mL/well EBS/0.2% BSA/0.1% glucose. The cells were then incubated at 37°C for 15 minutes to wash away extracellular inositol. This medium was removed and replaced with 0.45mL EBS/BSA/glucose containing unlabelled inositol (10mM) and lithium chloride (10mM). After 15 minutes incubation as before (to allow the 'cold' inositol to chase out the ^{3}H-inositol, and the lithium to inhibit phosphatases), agonists/antagonists were added as required in a volume of 50μL and the cells were incubated at 37°C for 20 minutes. Except in the case of two wells taken for protein analysis, 250μL cold 15% perchloric acid was then added per well to terminate the stimulation. The inositol phosphates were then extracted as described below.

2.2.2.6.3 Extraction of inositol phosphates and quantitation of turnover

The cells were scraped from the wells using the plunger of a 2mL plastic syringe and transferred to a 1.5mL Eppendorf tube. The wells were rinsed with 0.5mL distilled water and this rinse was also transferred. The samples were then centrifuged in a microfuge for 3 minutes at 6,500rpm and the supernatant, containing the inositol phosphates, transferred to a glass

tube. 1.5mL of a freshly-prepared mixture of 1:1 trichlorotrifluoroethane/tri-n-octylamine was then added to each tube and the samples vortexed thoroughly until the samples appeared milky. The tubes were then centrifuged (3 minutes, 3,000rpm) to separate the phases. 0.9mL of the aqueous upper phase was removed to a clean 1.5mL Eppendorf tube.

100μL of 10mM EDTA was then added and each tube mixed well. The samples were passed through polypropylene columns (with a sintered glass base disc, 1cm diameter) containing 0.5mL AG1X8 anion-exchange resin (previously equilibrated with distilled water) and eluted with 2X 2mL 1M ammonium formate/0.1M formic acid buffer. The eluates were collected into 5mL scintillation vials and 3mL scintillant (HydroLuma) was added to each vial. These were securely capped and shaken well to ensure mixing of the phases. The vials were counted for 10 minutes in a Canberra-Packard CA1900 liquid scintillation counter programmed to obtain DPM values.

2.2.2.7 ^{125}Iodination of aldosterone

Aldosterone was labelled with ^{125}I for use in radioimmunoassay using the Chloramine T/histamine method as detailed below. (Hunter et al, 1976)

Firstly, 400μL aldosterone (in the 3-methyl-mono-oxime form; a 0.725mg/mL solution in ethanol) was dried down in a glass tube under nitrogen and redissolved in 50μL dimethylformamide cooled to 10°C. The tube was maintained at 10°C in a water bath containing ice and to the redissolved oxime was added 10μL tributylamine (85μL stock diluted in 5mL dimethylformamide) and 10μL isobutylchloroformate (45.4μL stock diluted in 5mL dimethylformamide). The mixture was then vortexed and kept at 10°C for 20 minutes.

Next, the histamine was ^{125}I-iodinated. 10μL Na^{125}I (~1mCi) was put in a conical based glass tube and to this was added 10μL histamine (2.2mg in 10mL 0.25M phosphate buffer pH 7.4) and 10μL Chloramine T (50mg in

10mL 0.25M phosphate buffer pH 7.4). The tube was vortexed for 15 seconds and then 10μL sodium metabisulphite (120mg in 10mL 0.05M phosphate buffer pH 7.4) was added before vortexing again and cooling to 0°C on ice. The iodinated histamine was then reacted with the activated aldosterone as follows.

280μL cooled dimethylformamide was added to the activated aldosterone and vortexed. 50μL of the diluted aldosterone solution was then added to the ice cold iodination mixture. 10μL 0.2M NaOH was then added, the tube well vortexed and incubated at 0°C for two hours. After this time, 1mL 0.1M HCl and 1mL redistilled ethyl acetate were added to the ^{125}I-iodination mixture. The tube was capped, vortexed for 5 seconds and the upper (organic) layer was removed and discarded. 1mL 0.1M NaOH and 1mL potassium iodide (100mg in 10mL 0.5M phosphate buffer pH 7.4) were then added and the mixture vortexed before being extracted with ethyl acetate as described below.

250μL redistilled ethyl acetate was added to the ^{125}I-iodination mixture and the tube was capped and vortexed for 1 minute. The lower aqueous layer was removed to a second conical-based tube and the organic layer was transferred to a clean glass tube. The aqueous layer was extracted a further two times, and the organic layers combined.

The ^{125}I-iodinated aldosterone (aldosterone-3-CMO-(2-(^{125}I)-iodohistamine) was then purified by thin layer chromatography. The organic extract was applied in a narrow band approximately one inch from the bottom edge of a 200x200mm silica TLC plate (Merck; silica gel 60) and run in a solvent consisting of toluene:ethanol:acetic acid (75:24:1) for approximately two hours. The plate was then removed from the tank and allowed to dry before being wrapped in cling film and exposed to Kodak X-ray film (X-OMAT AR5) for 15-20 minutes. The position of the ^{125}I-aldosterone band was marked on the TLC plate. Two bands were observed; the lower band of which was known to be the immunoreactive compound,

with a r_f of 0.2-0.24. The silica containing this band was carefully scraped off and transferred via a glass funnel to a glass scintillation vial. The ^{125}I-aldosterone was eluted with 3mL ethanol for one hour at room temperature. The ethanol was then filtered through a glass Pasteur pipette plugged with a small piece of cotton wool and containing 2cm celite to remove the silica. The column was washed with 3X1mL ethanol. The final ^{125}I-aldosterone product was stored at -20°C.

2.2.2.8 Iodination of Angiotensin II

This ^{125}I-iodination provided labelled AII for use in ligand binding studies, and was also carried out using the Chloramine T method. (Dusterdieck + McElwee, 1971)

10µL of 10mg/mL AII was mixed with 10µL 0.5M phosphate buffer pH 7.4 in a polypropylene tube. To this was added 10µL Na^{125}I; this was mixed before adding 10µL Chloramine T (10mg diluted in 10mL 0.5M phosphate buffer pH 7.4). This was again mixed and incubated for 60 seconds at room temperature. To stop the ^{125}I-iodination reaction, 10µL sodium metabisulphite (20mg diluted in 10mL 0.5M phosphate buffer pH 7.4) was added and mixed before adding 500µL 0.1M phosphate buffer (pH 7.4) and mixing the contents of the tube a final time.

The ^{125}I-iodinated AII was then purified by gel chromatography through a column of DEAE Sephadex A25 equilibrated with 0.1M phosphate buffer pH 7.4. The ^{125}I-iodination mixture was applied to the column, allowed to drain into it and then washed into the column with 1mL of buffer. Buffer was then pumped through the column using a low pulsatile peristaltic pump at a flow rate of 0.25mL/minute. Fractions were collected every 4 minutes for 5 hours into numbered polypropylene vials. 10µL aliquots from these fractions were counted for 60 seconds in a gamma counter to locate the ^{125}I peaks. The first to appear was the unreacted ^{125}I-iodine and was discarded. The second, which eluted at approximately 4 hours, corresponded to the mono-^{125}I-iodinated AII; the highest activity

fraction plus the two fractions on either side of the peak were collected and pooled; all other fractions were carefully discarded. The pooled fractions were made up to 50mL with 0.1M phosphate buffer pH 7.4 containing 0.1% (w/v) sodium azide and 0.5mL Trasylol (200 kallikrein inhibitor units/mL). 1mL aliquots were transferred into plastic tubes (LP4), capped and stored at -20°C. Specific activity of the ^{125}I-AII was routinely ~1000Ci/mmol.

2.2.2.9 Stimulation of cultured cells to monitor regulation of AT_1 receptor expression

Bovine adrenal zona fasciculata/reticularis cells were plated into 25cm^2 cell culture flasks at a density of 5X10^6 cells/flask, in 5mL growth medium. The cells were cultured as described in 2.2.1.2, with the medium being changed after 24 hours. On the second day after isolation, the medium was removed and the cells washed in sterile EBS. They were then incubated for 6 hours in serum-free medium (containing antibiotics as in 2.2.1.1) before commencing experiments.

After 6 hours, the serum-free medium was removed and 5mL of serum-free medium, either alone or containing agonists at the chosen concentrations was added. The cells were then incubated, normally for 48 hours. Medium was replaced every 24 hours. Experiments were designed so that all incubations finished at the same time. Therefore, if time points less than 48 hours were to be used, the agonists were added to the cells at the requisite time before the end of the experiment. At the end of the experiment the medium was removed and in some cases reserved for assay of cortisol secretion. The cells were then processed for RNA extraction as detailed in 2.2.3.2.

2.2.3 Molecular biology methods

2.2.3.1 DNA isolation from tissue

DNA was isolated from bovine spleen using a method based on a protocol (Sambrook *et al.* 1989) for preparing high molecular weight DNA. 250mg of bovine spleen (obtained fresh, on ice, from the local abattoir within 30 minutes of slaughter) was minced finely in cold PBS and homogenised (30 seconds, Polytron homogeniser, followed by one pass in a Dounce homogeniser). Remaining particulate matter was pelleted by a short spin (30 sec, 1000rpm) and the supernatant centrifuged at 200g for 10 minutes at 4°C to pellet the nuclei. These were resuspended in 3mL digestion buffer (50-mM Tris HCl; pH8.0, 100mM EDTA, 100mM NaCl, 1%(v/v) SDS), 175µL 10mg/mL proteinase K was added and the tissue incubated at 55°C for 4 hours. 200µL of 20µg/mL DNase-free RNase was then added and incubated at 37°C for a further hour, after which 375µL 2M β-mercaptoethanol and 6mL phenol were added and the tube contents mixed on a vertical rotator for 15 minutes. The phases were separated by centrifuging at 3000rpm for 10 minutes (Heraeus Omnifuge) and the aqueous phase transferred, along with the interphase, to a fresh tube. This procedure was then repeated, but without the addition of β-mercaptoethanol. 3mL of phenol and 3mL choroform were then added to the aqueous phase and rotated as before for 5 minutes, after which the phases were separated as before. The aqueous phase was then transferred to a clean tube (leaving behind the interphase) and 6ml chloroform was added, mixed and the aqueous phase separated as before. The DNA was precipitated by the addition of 6mL isopropanol and inverting several times. The DNA was pelleted by centrifuging at 500g for 10 minutes and resolubilised in 3mL TE (10mM Tris.HCl; 1mM EDTA pH 8.0) by incubating at 37°C overnight. After this the DNA was reprecipitated by adding 0.5 volumes 6M ammonium acetate and 2 volumes isopropanol and mixing well. The tube was centrifuged as previously to pellet the DNA, which was then washed with 1.8mL 70% ethanol followed by air drying

briefly. The pellet was finally resolubilised in 3mL TE pH8.0 by warming at 37°C for 30 minutes before being analysed for integrity (by running an aliquot on a 0.7% agarose 0.5X TAE gel) and quantitated by measuring absorbance at 260/280nm. (1OD at 260nm = 50µg DNA; ratio 260:280 should be 1.6-1.8).

2.2.3.2 Agarose gel electrophoresis of nucleic acids

Generally, the quality of nucleic acids after PCR, assembly of cloning vector, RNA isolation etc. was checked by electrophoresis through a 0.7% (w/v) 0.5X TAE agarose gel containing 0.5µg/mL ethidium bromide, to allow visual detection under UV light. (Sambrook et al, 1989) A small amount of sample was mixed with loading buffer (0.25% (w/v) bromophenol blue, 40% (w/v) sucrose, 60mM EDTA pH 8.0). The gel was prepared by melting 0.8g agarose (general molecular biology grade) in 75mL distilled water using a microwave oven, cooling to approximately 50°C, adding 0.75mL 50X TAE (Tris-acetate-EDTA buffer : when 1X = 0.04M Tris.acetate; 0.001M EDTA) and 3.75µL 10mg/mL ethidium bromide and pouring into a gel-forming tray (apparatus was either Pharmacia GNA100 or Bio-rad wide mini sub-cell). A comb with the appropriate number of wells was placed in the tray and the gel was left for 30 minutes to set. Once set, the gel was transferred (with tray) to a gel-running tank containing sufficient 0.5XTAE buffer to cover the gel. The samples were loaded into the wells and the electrophoresis was run at constant voltage (100V) for approximately 45 minutes, or until the blue dye had migrated half way down the gel, submerged in 0.5X TAE buffer. The nucleic acids could then be visualised by viewing the gel on a Spectroline UV transilluminator and could be photographed (Polaroid film) if required. λ DNA size markers were also run on each gel. When running RNA samples, the tank, tray and comb were treated with 3% (v/v) hydrogen peroxide for 15 minutes to destroy any contaminating RNAses; the gel and running buffer were prepared using RNAse-free water.

2.2.3.3 DNA quantitation by fluorimetry

This method was used for accurate quantitation of DNA. It uses a Hoefer TKO 100 fluorimeter and is based upon the fact that DNA fluoresces in the presence of Hoechst 3328 dye (method as per Hoefer manual).

After warming up for 30 minutes, the scale on the fluorimeter was adjusted to 50%. 2mL of TNE + dye mix (0.2µg/ml Hoechst 3328 Dye, 10mM Tris.HCl; pH7.4, 1mM EDTA, 0.2M NaCl) was placed into the cuvette, mixed by inversion, the sides of the cuvette were wiped and it was then placed in the fluorimeter. The fluorimeter was then zeroed. This step was repeated several times until consistent readings were obtained (+/- 5 units). The DNA standard was then set : 2mL of TNE + dye was placed in the cuvette and 2µL of 250ng/µL λ DNA was added and mixed well by inversion. A reading was then taken and the scale knob adjusted to read 250. This step was then repeated, using fresh dilutions each time, until consistent readings of 250 +/- 5 units were obtained. The machine was now ready to be used to measure the DNA concentration of samples. 2mL TNE + dye mix was placed in the cuvette, 2µL DNA sample added and the two mixed well, again by inversion. A reading was then taken. Readings over 700 units were deemed to be outside the linear range, and the sample was diluted before being re-read. More than one reading was taken for each sample to ensure consistent results were obtained.

2.2.3.4 Restriction enzyme digestion

2.2.3.4.1 General restriction digest conditions

DNA was digested at a concentration of 0.1-0.5µg/mL to a four-fold enzyme/time excess (twice the theoretical enzyme units needed to complete the digest (1 unit/µg DNA in 1 hour) and twice the time needed). Digests were carried out in the buffer recommended by the manufacturer (supplied with the enzyme) at 37°C. Enzyme never comprised more than 10% (v/v) of

the reaction mix to avoid glycerol interference with digestion. At the end of the digestion period (normally 2 hours) 0.5μg DNA was checked on an agarose gel to monitor completeness of the reaction. Reactions were stopped by addition of a 10mM EDTA excess (2.5μL 0.4M EDTA pH 8.0 per 50μL reaction) and heat-inactivation at 65°C for 10 minutes. (Sambrook et al, 1989)

2.2.3.4.2 Preparation of DNA size markers

These were prepared from λ phage DNA by digestion with EcoRI and HinDIII using the following reaction conditions : 200μL of λDNA (0.25μg/μL) was digested with 25 Units each of EcoRI and HinDIII (each 10U/μL) in Boehringer reaction buffer B, in a total reaction volume of 250μL. Digestion was at 37°C for 5 hours. After this time 2.5μL was checked on an agarose gel for completion of the digest. If the reaction was complete, 12.5μL 0.4M EDTA was added and the reaction was heat-inactivated at 65°C for 10 minutes. The DNA was then precipitated by the addition of 125μL 6M ammonium acetate and 750μL isopropanol and spun for 5 minutes at maximum speed in a microfuge. The pellet was washed with 180μL 70% ethanol and briefly air dried before reconstituting in 167μL TE and 33μL 6X sample loading buffer. These markers gave fragments of DNA of the following sizes : 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, 125 base pairs.

2.2.3.5 Polymerase chain reaction
(Sambrook et al, 1989)

The polymerase chain reaction was used to amplify the coding region of the AT_1 receptor gene (a fragment of 1077bp in length) from bovine DNA. The primers were designed to amplify the entire coding region of the AT_1 receptor (this was possible as there are no introns in the coding sequence) and had EcoRI (forward) and BamHI (reverse) restriction sites incorporated into them to permit generation of 'sticky ends' for insertion of the AT1

fragment into the cloning vector. A positive control reaction amplifying G3PDH from bovine DNA (primers : forward : 5'-ACCACAGTCCATGCCATCAC-3'; reverse : 5'-TCCACCACCCTGTTGCTGTA-3') was also used. During optimisation of the AT_1 PCR procedure the following were varied : annealing temperature (64, 65, 66°C); starting amount of DNA (0.5, 0.1, 0.02 and 0.01μg); number of cycles (30, 32 and 35) and type of DNA polymerase (Taq and ULTma, a proof-reading enzyme). The conditions chosen were : annealing temperature 65°C; initial DNA 0.02μg; 32 cycles and Taq polymerase. ULTma, although more accurate, proved unreliable under all conditions tested, possibly due to excessive 3'-5' exonuclease activity and lack of processivity.

General reaction conditions were as follows. 20ng bovine genomic DNA was amplified in a 25μL reaction containing 1μM of each of forward 5'-(TGGAATTCAAAATGATCCTCAACTCTTCC)-3' and reverse 5' (CTGGGATCCGGTTTCAAGTGTGTTACTCAACC)-3' primers, 0.2mM each dATP, dCTP, dGTP and dTTP, 1mM $MgCl_2$ and 0.25 Units Taq or ULTma polymerase (added after initial heat denaturation : 'hot start' conditions). The reaction buffer was 10mM Tris.HCl pH 8.3, 50mM KCl, 0.1mg/mL gelatine. 25μL light mineral oil was layered on top of each reaction tube and the tubes were then transferred to a Hybaid Omnigene PCR machine. The reactions were heat denatured at 94°C for 5 minutes before proceeding with a run of 32 cycles of denaturation (94°C, 1 minute), annealing (65°C, 1 minute) and extension (72°C, 1 minute, 30 seconds) followed by one cycle of 72°C for 10 minutes and 25°C for 1 minute. The PCR reactions were then extracted by the addition of 50μL chloroform, mixing and centrifuging to separate phases. The aqueous phase was transferred to a clean tube; 5μL of this was used for analysis on an agarose gel to check that amplification of the correct fragment size of DNA had occurred. The AT_1 PCR reaction yielded a band of 1114bp while the control GAPDH reaction yielded fragments of 700bp and 300bp. The remainder of the reaction was stored at

4°C until purification. Product from multiple reactions was pooled. To investigate the effect of purification of fragment on cloning efficiency, two different purification strategies were used; the AT_1 PCR product was therefore split into two pools, AT_1 ① and ②. Pool ① was extracted with successive volumes (1 X reaction volume) phenol, phenol+chloroform and chloroform, spinning each time (2 minutes, full speed in a microfuge) to separate phases and taking the aqueous phase to a clean 1.5mL tube. The DNA was then precipitated by the addition of 0.5 volume 6M ammonium acetate and 2 volumes isopropanol and incubating at -20°C for 30 minutes, followed by spinning for 5 minutes at full speed in a microfuge (4°C). The pelleted DNA was then washed in 70% ethanol, air dried and resuspended in 17.6μL water for the Klenow Kinase Ligase (KKL) procedure described in 2.2.3.6.

Pool ② was purified by passing through an Amicon microconcentrator, as in the second part of protocol 2.2.3.8.1. The concentrated DNA was made up to 17.6μL with sterile water for the KKL procedure.

2.2.3.6 Klenow kinase ligase procedure

This procedure was used to concatemerise the PCR product. This generated restriction sites in the middle of the molecule rather than at the ends, and therefore increased the efficiency of the restriction enzymes used (Lorens, 1991). DNA purified after PCR was resuspended in 17.6μL of sterile water and the following were added : 2.5μL 10X KKL buffer (300mM Tris HCl pH 7.8, 100mM $MgCl_2$, 100mM DTT, 5mM ATP), 0.5μL dNTP mix (as for PCR), 1.0μL Klenow polymerase (5U/μL), 0.4μL T4 polynucleotide kinase (10U/μL) and 2.0μL T4 DNA ligase (1U/μL). The reaction was incubated at 25°C for 2 hours, then heat-inactivated for 10 minutes at 70°C. 1.0μL was checked on an agarose gel to confirm that concatemerisation had

occurred, after which the product was digested with EcoRI and BamHI restriction enzymes as below.

2.2.3.7 Restriction digest of PCR product and vector.

Either 25μL KKL reaction mix (AT_1 ① and ②) or 25μL 0.2μg/μL cloning vector (pSP72poly4) were digested with EcoRI and BamHI to generate compatible 'sticky ends'. The DNA was mixed with 5μL 10X Boehringer restriction buffer B, 2.5μL of each of EcoRI and BamHI (10U/μL) and 15μL sterile water. Digests were incubated for 2 hours at 37°C; 2.5μL was checked on an agarose gel for completeness of digestion.

100ng DNA was reserved from the AT_1 pools at this stage for use in ligations (AT_1 ① and ② 'pre'). AT_1 ① and ② DNA was purified by preparative electrophoresis and gel extraction using the Amicon system, as described in section 2.2.3.8.

Vector DNA was precipitated by addition of 0.5 volumes 6M ammonium acetate and 2 volumes isopropanol and incubating at -20°C for 30 minutes, centrifuging, washing and resuspending in 25μL TE. This was then purified by preparative electrophoresis and Qiaex extraction as described in 2.2.3.8.

At this point the AT_1 and vector DNA was quantitated by fluorimetry (2.2.3.3) and 50ng run on an agarose gel to check integrity. All DNA samples were stored at 4°C until use.

2.2.3.8 Purification of AT_1 insert and vector DNA for cloning

2.2.3.8.1 Preparative electrophoresis

Both the PCR product and the vector into which the PCR product was to be inserted were purified using this method. The DNA was electrophoresed on a high grade, low melting point agarose (Seachem GTG

enzyme grade); a 75mL 0.5X TAE gel was used as described in 2.2.3.2, using a 5-tooth comb enabling large volumes (30μL) to be run. Before use the tank, gel tray and comb were washed with neutral detergent and treated with 3% (v/v) hydrogen peroxide to ensure removal of nucleases. The DNA sample (after KKL procedure and/or restriction digest to generate 'sticky ends') was loaded into a single slot of the comb and run at 50-60mA until the bromophenol blue in the loading buffer had migrated halfway up the gel. The gel was then placed under long-wave (312nm) UV light, the required band sliced out with a sterile scalpel, placed into a preweighed, sterile Eppendorf tube and the weight of agarose removed determined. At this point the gel was photographed to show the position of the excised bands; doing this before excision of the fragments can damage the DNA and lead to reduced ligation and transformation efficiencies. The DNA was then eluted from the agarose fragment in one of two ways, each detailed below.

2.2.3.8.2 Extraction of DNA from agarose : Qiaex resin

This method followed the manufacturer's (Qiagen) protocol. 300μL of solubilisation buffer QX1 was added to the Eppendorf tube containing the excised gel fragment for each 100mg of gel. 10μL of well-vortexed Qiaex suspension was then added and the agarose solubilised by incubating at 50°C for 10 minutes. The sample was vortexed every two minutes to keep the resin in suspension. The tube was then centrifuged in a microfuge at full speed for 30 seconds. The resultant pellet contained the DNA bound to the Qiaex resin. This was resuspended by the addition of 500μL of QX2 buffer and vortexing, followed by centrifugation to pellet the resin. This step was repeated once with buffer QX2 and twice with buffer QX3. All traces of supernatant were then removed and the pellet was briefly air dried. The DNA was eluted from the Qiaex resin by the addition of 20μL TE, resuspension by vortexing and incubation for 5 minutes at room temperature, with periodic vortexing to keep the resin in suspension. The

sample was then centrifuged as before and the supernatant carefully transferred to a fresh tube. The elution procedure was then repeated and the eluates combined. The purified DNA was then checked for integrity on an agarose gel and quantified by DNA fluorimetry (2.2.3.3).

2.2.3.8.3 Extraction of DNA from agarose : Amicon columns

This method used Micropure inserts in Microcon microconcentrators to extract DNA from agarose slices and to wash and concentrate the product as per the manufacturer's instructions. The excised agarose fragment from the preparative electrophoresis was minced with a sterile scalpel until only small (1-2mm) pieces remained. 100μL TE pH 8.0 was then added to the tube and a peroxide-treated Eppendorf pestle was used to homogenise the gel into a slurry. The gel slurry was transferred into a Micropure insert inside an assembled Microcon microconcentrator unit. The Eppendorf was rinsed with 100μL TE and this was also transferred to the insert. The unit was then capped securely and spun in a microcentrifuge for 10 minutes (room temperature, full speed). The liquid had then passed through the insert, but was retained above the membrane of the microconcentrator.

The insert was then discarded and the Microcon concentration unit transferred to a clean Amicon tube and filled with 450μL TE. The apparatus was then capped and spun as before, with an additional spin if liquid remained above the membrane after the first spin. This washing step removed any enzymatic impurities released from the agarose with the DNA. The wash step was then repeated. The high purity DNA was recovered from the Amicon membrane as follows : 10μL TE was layered gently on top of the membrane, the Microcon apparatus was removed from the tube and transferred, upside-down, to a clean Amicon tube. The DNA was spun down (30 seconds, top speed) and diluted with TE to a volume of 40μL (or less if, for example, the DNA was to be subjected to KKL procedures).

The integrity of the recovered DNA was checked by agarose gel

electrophoresis (2.2.3.2), and fluorimetry (2.2.3.3) was used to accurately quantitate the DNA. The recovered DNA was stored at 4°C until use.

The second half of this protocol i.e. the concentrating of DNA (from volumes of up to 500μL) could be used independently of the gel extraction protocol. Dilute or impure DNA was placed directly into a Microcon apparatus, spun down (10 minutes, full speed) to concentrate the DNA, the DNA was then washed twice as described and the concentrated DNA recovered as above.

2.2.3.9 Ligation of AT_1 insert into pSP72poly4 vector

Ligations were carried out using several different ratios of insert (AT_1 pools ① and ② 'pre' and 'post') to vector. AT_1 DNA, purified in different ways as described in 2.2.3.5, was ligated into the vector at molar ratios of 5:1 and 20:1. 10ng vector DNA (2.5kb long) were used in each ligation which meant that the amounts to be used for 5:1 and 20:1 ratios were 20ng and 80ng insert (1kb) respectively. The two DNA samples were mixed with 4μL 5X ligation buffer (from BRL), 1μL 10mM ATP, 1 unit T4 DNA ligase and made up to a volume of 20μL with sterile water. A vector self-ligation control was included, where the insert was replaced with water; a non-ligated control (no ligase) was also included. The reactions were incubated at room temperature for 4 hours and then used in transformations.

2.2.3.10 Preparation of competent bacteria

E.coli strain DH5 (Hanahan, 1983) was made competent using the method of Chung *et al.* (1989). Briefly, 1mL of a fresh overnight culture of bacteria was inoculated into 100mL L-broth supplemented with 10mM $MgSO_4$ and 0.2% (w/v) glucose. This was incubated at 37°C, 225rpm for 2 hours to give an A600 of 0.2-0.3. The cells were then cooled to 4°C on ice-water and centrifuged for 5 minutes at 1000g, 4°C using a pre-cooled swing-

out rotor. The cells were resuspended by the addition of 10mL of ice-cold Miller transformation solution (L-broth containing 10% (w/v) PEG 8000, 5% (v/v) DMSO, 50mM $MgCl_2$ at a final pH of 6.5) and gentle swirling. At this stage, the cells were competent. Aliquots of 440μL were snap-frozen in 1.5mL Eppendorf tubes in a dry ice-ethanol bath and stored at -70°C until use.

2.2.3.11 Transformation of bacteria with cloning vector

Competent cells were thawed slowly on ice-water and gently resuspended. AT_1 PCR product, ligated to pSP72poly4 as in 2.2.3.9, was diluted to 100μL with TE (from a ligation reaction volume of 20μL). 40μL of the diluted reactions were pipetted into chilled 15mL polypropylene tubes. 100μL of competent cells were added to each tube and swirled to mix; the tubes were then incubated for 30 minutes at 4°C. 0.9mL of prewarmed SOC medium (2%(w/v) bactotryptone, 0.5%(w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) was then added, the tubes securely capped and incubated for 1 hour at 37°C, 225 rpm, to allow expression of ampicillin resistance. 100μL transformation mix was then plated out onto L-agar ampicillin plates (with 100μg/mL ampicillin); the remaining mix was centrifuged at 3000rpm for 10 minutes, 700μL of the supernatant removed and the remainder used to resuspend the pelleted cells; these were plated out in the same manner. The plates were incubated, upside-down, overnight at 37°C. Colonies on each plate were then counted; if there was an increase in numbers in the AT_1 ligations compared to the self- and non-ligated controls, colonies were picked and processed for minipreps (see 2.2.3.11). Table 2.1 shows the transformation efficiencies resulting from the different ligations performed.

2.2.3.12 Alkaline lysis minipreps of DNA

This rapid method of assessing whether the plasmid inserted into the competent cells contained the required insert was based on that of Birnboim and Doly (1979).

2mL of L-broth containing 0.2% (w/v) glucose and 100μg/mL ampicillin was inoculated with a single bacterial colony from a plate containing putative transformants and grown to saturation overnight at 37° C. 1.5mL of the culture was then transferred to an Eppendorf tube and spun in a microfuge at full speed for 20 seconds to pellet the cells. The remaining culture was kept at 4°C. The pellet was resuspended in 100μL of 'lysis buffer' (25mM Tris.HCl pH8.0, 10mM EDTA, 10%(w/v) glucose, 2mg/ml lysozyme) and left at room temperature for 10 minutes. 200μL fresh sodium hydroxide/SDS solution (0.2M-NaOH, 1%(w/v) SDS) was then added and mixed immediately by vortexing gently and placed on ice for 5 minutes. 150μL 3M potassium acetate pH 4.8 was then added to the suspension and vortexed vigorously for 10 seconds to ensure mixing was complete. The tube was then placed on ice for a further 5 minutes and spun for 1 minute at full speed in a microfuge to pellet the cell debris and chromosomal DNA. The supernatant was then transferred to a clean tube; 0.9mL isopropanol was added and the tube left at -20°C for 15 minutes to precipitate nucleic acids. The solution was spun for 2 minutes at room temperature to pellet the plasmid nucleic acids, which were resolubilised in 40μl TE (10 minutes, 37° C). To the resolubilised nucleic acids 20μl 6M ammonium acetate and 120μL isopropanol was added, the solutions were mixed by inversion and incubated at -20°C for 15 minutes. The nucleic acids were spun down as before and the pellet washed with 180μL 70% ethanol and air dried for 10 minutes, before resuspending in 20μL TE containing RNAse A (50μg/mL). Yield of DNA was usually 1-5μg. Of this 2μL was digested with appropriate restriction enzymes (to excise the insert from the Table 2.1 shows the frequency of successful ligations for the different AT_1 pools used. If the

Ligation	Transformants/μg DNA	Successful ligations / 5
non-ligated	5.9×10^4	
vector self-ligated	3.6×10^4	
AT_1 ① 'pre' 5:1	5.6×10^4	0
AT_1 ① 'pre' 20:1	1.1×10^4	0
AT_1 ① 'post' 5:1	1.4×10^5	0
AT_1 ① 'post' 20:1	3.9×10^4	1
AT_1 ② 'pre' 5:1	2.6×10^4	0
AT_1 ② 'pre' 20:1	1.6×10^3	0
AT_1 ② 'post' 5:1	8×10^4	5
AT_1 ② 'post' 20:1	6×10^4	5
control 2:1	1.2×10^5	
control 5:1	2.1×10^5	

N.B. 1. The competence of the *E.Coli* cells was checked and found to be sufficient at 7.4×10^6 transformants/μg DNA.

2. The terms 'pre' and 'post' refer to AT_1 DNA taken before and after gel purification of the insert respectively.

3. The control insert was a 2.7kb EcoRI-BamHI fragment excised from another plasmid, pR2P4.3.

Table 2.1

Transformation efficiencies and frequencies of successful ligations for the two AT_1 pools before and after gel purification of the AT_1 PCR product.

presence of the insert was confirmed, other restriction digests were performed to confirm that the DNA inserted contained the right sequence. Large quantities of bacteria containing the AT_1 plasmid could then be prepared as described below.

2.2.3.13 Large scale plasmid preparation and purification of DNA

This method was based on that of Birnboim and Doly (1979) and was used for preparing large quantities of high-quality DNA from the restriction-mapped positive AT_1 clones. The resultant DNA was of sufficient standard to be used for sequencing and preparation of probes for Northern blots.

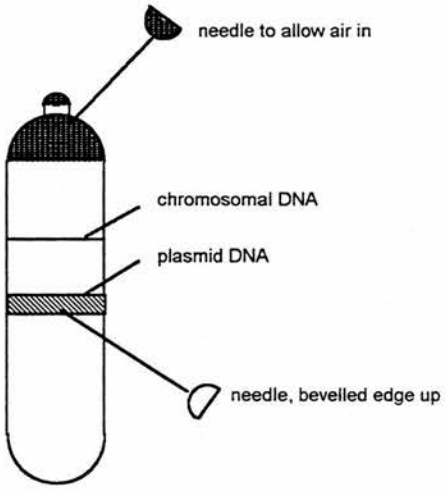
10mL L-broth supplemented with 0.2% (w/v) glucose and 100μg/mL ampicillin was inoculated with 200μL culture reserved from a positive miniprep and grown to saturation overnight at 34°C. This culture was then used to inoculate 500mL L-broth supplemented as above and the new culture was grown for 16-24 hours at 34°C, 250rpm in an orbital shaker. The lower temperature was used to facilitate plasmid replication over bacterial division. The cells were harvested by centrifugation for 10 minutes, 4°C, 5000rpm and resuspended in 4mL glucose/Tris/EDTA solution (25mM Tris HCl pH 8.0, 10mM EDTA, & 50mM glucose). This solution was transferred to a clean tube and 2mL fresh lysozyme solution (10mg/mL) added and mixed gently. The tube was left on ice for 15 minutes. 10mL freshly prepared NaOH/SDS solution (0.2M NaOH, 1%(w/v) SDS) was then added and mixed gently until a clear, homogeneous solution was obtained. The tube was then left for a further 10 minutes on ice before adding 7.5mL potassium acetate solution (pH 5.5; 3M potassium acetate, 1.18M formic acid). The solution was incubated for a final 10 minutes on ice and then centrifuged for 15 minutes, 4°C, 13,000rpm to pellet the bacterial DNA.

The supernatant (containing plasmid DNA) was carefully decanted into a clean tube and the DNA precipitated by the addition of 1 volume of cold isopropanol and incubation at -20°C for one hour. The mixture was then

centrifuged for 10 minutes (4°C, 13,000rpm) to pellet the DNA. This was then dissolved in 9mL TE before being purified by caesium chloride gradient centrifugation.

The 9mL DNA solution was transferred to a 15mL polypropylene tube containing exactly 9.9g CsCl. After warming gently to effect complete solubilisation, 0.45mL 10mg/mL EtBr was added. This solution was then carefully transferred to an 11mL Beckman polyallomer quick-seal centrifuge tube and balanced to within 0.05g of another sample tube or balance tube (of TE/CsCl/EtBr) and heat-sealed. The tubes were then spun in an NVT65 rotor for 24 hours, room temperature, 55,000rpm.

After centrifugation, the plasmid DNA was visible as a reddish band which fluoresced under UV light. The band was removed using a 19G needle and 2mL plastic syringe as shown below :



The DNA solution removed from the gradient was then purified from contaminating CsCl and EtBr. The solution was made up to 2.5mL with TE and passed through a NAP-25 column. It was then eluted into a clean 15mL tube by passing 3.5mL TE through the column. Intercalated EtBr was then removed by extracting the solution successively with 1 volume phenol+chloroform and 1 volume chloroform. To maximise yield, the

phenol+chlorofrom was extracted with 1 volume TE and this was combined with the aqueous phase from the chloroform extract. The DNA was then precipitated with 0.1 volume 3M sodium acetate pH 5.5 and 2 volumes cold isopropanol, incubating for 1 hour at -20°C. The tube was centrifuged for 10 minutes, 4°C, 4000rpm to pellet the DNA, which was washed in 1mL 70% (v/v) ethanol and spun again. The pellet was then air dried for 10 minutes and resolubilised in 0.5mL TE, then transferred to a 1.5mL Eppendorf tube and stored at 4°C. 1μL was checked on an agarose gel to examine integrity; an accurate quantitation was obtained by fluorimetry. The concentration was adjusted to 1μg/μL and the correct identity of the plasmid checked by restriction mapping similar to that performed after minipreps.

2.2.3.14 Sequencing of AT_1 receptor clones

To ensure that the clones of the AT_1 receptor generated were entirely homologous with the published sequence, it was necessary to sequence them. This was accomplished by the Sanger (Sambrook et al, 1989) dideoxynucleotide method, using the Sequenase kit from USB. Multiple primers were used at sites throughout the coding sequence in order to generate complete sequence on both strands (the technique of 'primer walking'). The primers used are listed below :

<u>forward i.e. 5'-3'</u>	
5'-CTGAGAGTGCACCATATG-3'	pSP72 forward
5'-CTGGAATTCAAAATGATCCTCAACTCTTCC-3'	PCR EcoRI
5'-GTGGGCTGTCTACACTGC-3'	AT_1 ①
5'-GAGAATACCAATATCACC-3'	AT_1 ③
5'-GATGTGTTAATTCAGTTGG-3'	AT_1 ⑤
<u>reverse i.e. 3'-5'</u>	
5'-CAATACGCAAACCGCCTC-3'	pSP72 reverse

5′-CTGGGATCCGGTTTCAAGTGTGTTACTCAACC-3′	PCR BamHI
5′-CTTCAATTTTACAGTCAC-3′	AT_1 ②
5′-GGATTCGTAATGGAAAGC-3′	AT_1 ④
5′-AGGTTGAAACTGACACTG-3′	AT_1 ⑥

For most of the reactions, dGTP was used. Occasionally, however, it was necessary to sequence using its analogue dITP to resolve compressions occurring in the sequence due to CG repeats. The reaction is described for one primer but normally up to four primer reactions were run simultaneously.

The double-stranded DNA was denatured as follows. For each sequencing reaction (i.e.use of one primer) 5μg AT_1 DNA was needed. To this, 0.1 volume 2M NaOH/2mM EDTA solution was added and the mixture was incubated at 37°C for 30 minutes. 0.1volume 3M sodium acetate and two volumes 100% ethanol were then added, mixed and the samples incubated at -70°C for 30 minutes. The samples were centrifuged at 4°C for 30 minutes and the pelleted DNA was then washed by the addition of 0.5mL 70% ethanol, inversion and a further spin for 5 minutes. The wash was also discarded, the pellet air dried for 5 minutes and resuspended in 7μL sterile water.

The sequencing reactions were performed as detailed in the Sequenase kit protocol. Briefly, 5μg (7μL) denatured template DNA (from the AT_1 clone) was mixed in a 1.5mL Eppendorf tube with 2μL 5X reaction buffer (200mM Tris.HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl) and 1μL primer (1pmol/μL). The tube was heated to 65°C for 2 minutes to anneal the primers to the DNA and left to cool slowly to <35°C over 30 minutes. 2.5μL of each termination mixture (8μM ddA,C,G/I orTTP; 80μM dA,C,G/I and TTP in 50mM NaCl) was pipetted into a 1.5mL Eppendorf tube and prewarmed to 37°C. To the annealed DNA, 1μL 0.1M dithiothreitol, 2μL labelling mix (1.5μM dG/I,C,TTP), 0.5μL [^{35}S]-dATP and 2μL 1:8 diluted

Sequenase enzyme (buffer is 10mM Tris.HCl pH7.5, 5mM DTT, 0.5mg/mL BSA). After each addition, the tube contents were mixed by pipetting up and down. The reaction was incubated for 2-5 minutes at room temperature, after which 3.5μL of the labelling reaction was transferred to each of the termination tubes. The termination reactions were allowed to proceed for 5 minutes at 37°C, after which they were stopped by the addition of 4μL stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The reactions were stored at -20°C until being run out on a polyacrylamide gel.

Gradient gels were formed between 600mmx150mm glass plates to a width of 0.7mm. The plates used were washed with distilled water and phosphate-free detergent, water alone and then ethanol to ensure cleanliness and absence of grease. The inner side of one plate was siliconised to make separation of the plates after running the gel easier. The plates were then taped together and the gel poured. The gradient to be used was 0.5-4.0X TBE (Tris-borate-EDTA; when 0.5X = 0.045M Tris.borate; 0.001M EDTA) in a 6% acrylamide gel. The 4.0X TBE solution also contained 5% sucrose to allow for easier formation of the gradient. Both solutions contained 46% (w/v) urea to denature the DNA. 8mL 4.0X TBE gel was prepared first (polymerised by the addition of 16μL 25% (w/v) ammonium persulphate and 8μL TEMED. The 0.5X TBE gel was prepared to a volume of 60mL, using 120μL and 50μL of ammonium persulphate and TEMED respectively to polymerise. To form the gradient, 7mL of the 0.5X TBE gel was mixed with the 4.0X TBE gel and poured between the plates. As it settled to the bottom, the remainder of the 0.5X TBE gel was poured slowly between the plates until it reached the top. At this point a 24-well comb (treated as were the plates) was inserted into the gel and the plates secured together by means of bulldog clips. The gel was left for one hour to set. After this time, the clips were removed, the comb removed and the top of the gel washed gently with distilled water to remove any crystals of urea. The tape was then taken off and the gel

transferred to the running tank (BRL). 0.5X TBE buffer was then added to the bottom and top reservoirs, the comb replaced and the tank sealed up.

The samples were thawed if necessary and then heated to 75°C for 2 minutes to denature the DNA. They were then placed immediately on ice and kept there while loading (2μL per sample) onto the gel. The gel was run for 13,000volt-hours, until the xylene cyanol dye front had just reached the bottom of the gel plate.

The gel was then removed from the tank and the plates separated. The gel, still attached to the non-siliconised plate, was fixed in a tank containing 10% (v/v) glacial acetic acid and 10% methanol in distilled water for 15 minutes. After this, the gel was removed from the fix solution, drained and then transferred to a piece of Whatman 3MM filter paper before being dried for 2 hours at 80°C. The dried gel was then laid down to Kodak XAR-5 film overnight at room temperature to obtain an autoradiograph of the sequence information.

The data were entered using a DNAstar (Madison, Wisconsin) automated sequence reader and analysed using DNAstar software. Matching sequences from different primers and sequences from the opposite strand were also put together using the Sequencing Project Manager and the Align program of the DNAstar software.

2.2.3.15 RNA isolation from cells in culture

It was ensured that all solutions, plastics and glassware used when working with RNA were RNAse free. The method used was an adaptation of the RNAzol method described by Chomczynski & Sacchi (1987). Briefly, cells in 25cm^2 flasks were rinsed with EBS to remove any traces of growth medium and then lysed by the addition of 1mL of RNAzol. The cells were then scraped off the flask, pipetted up and down several times to ensure the solubilisation of the RNA and then transferred to clean 1.5mL Eppendorf tubes and kept on ice. 100μL chloroform was then added and the tubes

vortexed for 15 seconds and left on ice for 5 minutes. The phases were separated by spinning at full speed in a refrigerated microfuge for 15 minutes, after which the aqueous phase was transferred into a clean tube. The RNA was precipitated by adding 1 volume isopropanol and incubating at 4°C for 30 minutes. RNA was then pelleted by spinning at full speed in a refrigerated microfuge for 15 minutes. The pellet was washed with 1mL 70% ethanol, centrifuged again as before and briefly air dried before being solubilised in 100μL RNAse-free water. RNA samples were stored at -70°C. Before use in Northern blots, they were run on a 0.7% agarose gel to confirm their integrity, and quantified by measuring the A260 on a UV spectrophotometer (1OD unit = 40μg RNA; A260 : A280 ratio should ideally lie between 1.8 and 2.0).

2.2.3.16 Northern blotting

This method, for separating RNA samples by electrophoresis followed by permanent transfer to a nylon membrane and hybridisation with a labelled probe to a particular mRNA species, was adapted from that of Thomas (1983) and consisted of three stages, each described below.

2.2.3.16.1 Sample preparation and electrophoresis

Usually 25μg total RNA isolated from bovine adrenal cortex zfr cells in culture was analysed, but as little as 15μg could be analysed if yield was limiting. Samples were precipitated by the addition of 0.1 volume 3M sodium acetate pH 5.5 and 3 volumes absolute ethanol. The samples were incubated overnight at -20°C. A negative control (i.e. RNA which contained no AT_1 mRNA; total RNA from either cultured thyrocytes or Swiss 3T3 cells was used) was also prepared as were "copy controls". These were samples of negative control RNA spiked with small amounts of AT_1 receptor coding region DNA (the same DNA used to make the hybridisation probe). The

amounts chosen represented 2 and 20 copies of AT_1 mRNA per cell, based upon the fact that mRNA comprised 2% of total RNA, and the assumption that AT_1 mRNA was expressed at the low level of 1/100,000 mRNA molecules. In terms of weight of RNA this represented detection of 5 and 50pg RNA in a total sample of 25μg. RNA and DNA markers (5μg each of total RNA and λ DNA markers) were also processed. After incubation at -20°C, the samples were centrifuged for 15 minutes at full speed in a refrigerated microfuge to pellet the RNA. The pellets were washed by the addition of 180μL 70% ethanol, brief, gentle vortexing and spinning for 5 minutes as before. The RNAs were then air dried for 5 minutes and resuspended by the addition of 3.7μL RNAse-free water. 12.3μL denaturing mix was then added (for 20 samples : 54μL glyoxal, 32μL 0.1M phosphate buffer and 160μL DMSO, made fresh before use) to each sample, care being taken that the RNA was completely redissolved. The RNA samples were then denatured by heating at 50°C for 1 hour, followed by immediate cooling on ice. 4μL RNA loading buffer (10mM Na Phosphate; pH 7.0, 50% (v/v) glycerol, 0.4% (w/v) bromophenol blue) was then added and the samples loaded onto the gel.

A larger gel was used for the Northern blot electrophoresis than for the analytical gels, with a higher percentage of agarose. Prior to use, the tank, gel forming plates and comb were washed with neutral detergent and treated for 15 minutes with 3% (v/v) hydrogen peroxide to remove RNAses. The apparatus was then rinsed well with RNAse-free water and drained. A 1.2% (w/v) agarose gel (with no ethidium bromide, as this reacts with the glyoxal denaturing reagent), containing 10mM phosphate buffer, was poured into the tank between the gel forming plates and left to set. After setting, the gel was submerged in 10mM phosphate buffer pH 7.0. Recirculation of the buffer during the electrophoresis was necessary as the phosphate buffer ionises during electrophoresis, and the glyoxal dissociates from the RNA at pH>8.0.

The samples were then loaded onto the gel. A lane was left between the markers and the samples and controls to allow easy identification of sample position. Recirculation of the buffer was not started until the samples had fully entered the gel to avoid loss of the samples from the wells. The gel was run at constant voltage (100V) for 3 hours, after which time the bromophenol blue marker dye had migrated to within 1-2cm of the end of the gel. At the end of the run, the RNA was transferred to a nylon membrane by capillary transfer as described below.

2.2.3.16.2 Capillary transfer

The capillary wick was formed as follows. A large glass plate was balanced on top of a pyrex dish. Three pieces of Whatman 3MM were cut, the same width as the gel (15cm) and three times as long. A small amount of 20X SSC (3.0M NaCl, 0.3M Na citrate) was poured on the plate and the first piece of paper laid on top. Air bubbles were removed by rolling a glass pipette over the plate. This process was repeated with the other two pieces; the remainder of a 500mL bottle of 20X SSC was poured into the dish. The gel was then placed, upside-down, on top of the wick and air bubbles removed as before. The top left-hand corner of the gel was cut off to aid orientation. A piece of positively-charged nylon membrane the same size as the gel (15 X 15cm) was wetted in 2X SSC and gently placed on top of the gel, positively-charged side down, and air bubbles were again removed. Nine pieces of Whatman 3MM paper were cut to the same size as the gel. One piece was wet in 2X SSC and gently placed on top of the membrane, rolling out air bubbles as before. This was repeated with 5 more pieces, the other 3 were placed on top dry. The sides of the wick were covered with parafilm to ensure that transfer only took place through the gel and glass pipettes were placed on two opposite sides of the setup to support the transfer. Absorbent tissues were then placed on top of the Whatman paper (three double stacks of about 3cm each), a smaller glass plate was put on top

of these and weighted with a full 500mL bottle. Transfer was left to proceed overnight.

The next day the transfer setup was disassembled, the position of the wells was marked clearly on the membrane and the top left hand corner cut off (as on the gel). The membrane was rinsed briefly in 6X SSC and then air dried for 30 minutes before being baked at 80°C for 30 minutes to remove the glyoxal. The RNA was then covalently bound to the membrane by exposing it to 240mJ UV light at 312nm in a Bio-rad cross-linker.

To check that the RNA had been transferred and that it was still intact, and to allow detection of the size markers, the two lanes containing these samples were cut off from the blot and stained for 5 minutes in methylene blue (0.04% (w/v) in 0.5M sodium acetate pH 5.5). Destaining in distilled water revealed nucleic acids stained blue. If the correct pattern of bands was observed, the blot was then subjected to hybridisation as described below. Figure 2.3 shows the appearance of the stained DNA and RNA markers correctly resolved and transferred.

2.2.3.16.3 Hybridisation with specific probe fragments of DNA

The blot was wetted with 6X SSC and placed in a Techne hybridisation bottle. If more than one blot was to be hybridised, two blots could be placed in each tube. It was then prehybridised (blocked) in 20mL prehybridisation solution (50% (v/v) deionised formamide, 5x SSPE (when 20X = 3M NaCl, 0.2M Na phosphate pH 7.4, 0.02M EDTA), 5x Denhardt's solution (when 100X = 20mg/L Ficoll, 20mg/L polyvinylpyrrolidone, 20mg/L BSA), 0.5% (w/v) SDS, 1mM EDTA and 100µg/ml heat-denatured salmon sperm DNA) for 4 hours at 42°C. A Vogelstein random primed probe (2.2.3.17.1) or an asymmetric PCR probe (2.2.3.17.2) was made using either cloned bovine adrenal AT_1 receptor coding region DNA (1kb) or a 1.25kb fragment of rat β-actin cDNA. This was added to 20mL prehybridisation solution and hybridised to an unblocked piece of positively charged nylon

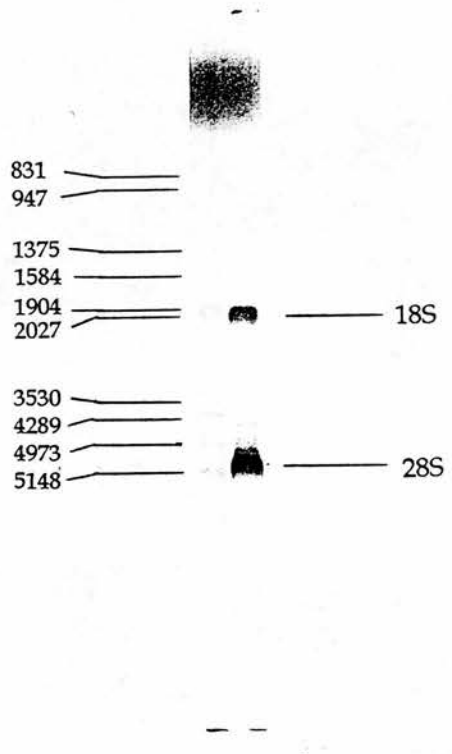


Figure 2.3

Marker lanes from a representative Northern blot stained with methylene blue to visualise nucleic acids. Lane 1 shows 5μg λ DNA markers, with the sizes indicated, and lane 2 shows the same amount of bovine adrenal RNA. The 18 and 28S ribosomal bands are indicated. The nucleic acids in both lanes are intact and transfer has occurred evenly, showing that the quality of the blot is sufficient for hybridisation to proceed.

membrane for 4 hours. The probe was then denatured by boiling for 10 minutes, cooling on ice and then the blot prehybridisation solution was replaced with the boiled probe solution and hybridisation was carried out overnight at 42°C.

Non-bound probe was then washed off the membrane using a progression of low and high stringency 50mL washes as follows.

- Low stringency : 2X SSPE, 0.1% SDS; 2 washes at room temperature followed by 2 washes of 15 minutes each at 65°C.
- High stringency : 0.2X SSPE, 0.1% SDS; 2 washes of 10 minutes at 65°C and 2 washes of 30 minutes at 65°C.

The blot was then briefly air dried and wrapped in cling film while still damp, before being laid down either to autoradiography (Kodak XAR-5 film, 2 enhancement screens, 72-96 hours at -70°C) or phosphorimaging (Biorad Molecular Imager CS or BI screens, 24-48 hours, room temperature).

If, after exposing the blot, it was wished to reprobe it (for example, if normalisation of AT1 receptor mRNA signal to a 'housekeeping gene' - β-actin - was required), the bound probe was stripped from the membrane. The blot was wetted with 2X SSC and placed in a hybridisation tube. It was then washed with a 50mL solution of 5mM Tris.HCl pH 8.0, 2mM EDTA pH 8.0, 0.1X Denhardt's solution and 0.1% SDS for 1 hour at 68°C. The blot was then air dried and either stored wrapped in cling film and a plastic bag or was wetted with 6X SSC to continue with hybridisation as described above.

2.2.3.17 Preparation of probes for Northern blots

2.2.3.17.1 Preparation of 'Vogelstein' random primed probe

This procedure for labelling specific fragments of DNA with ^{32}P for use in Northern blots was adapted from that of Hodgson and Fisk (1987).

50ng template DNA and 250ng random primers (Boehringer Mannheim) were made up to a volume of 10μL with TE and denatured at 100°C for 5 minutes, then

quenched on ice to avoid renaturation. The following reagents were added (in this specific order) : 5μL labelling buffer (0.5M Tris HCl; pH 6.9, 0.1M $MgSO_4$, 1mM DTT, 1mM dATP, 1mM dGTP and 1mM dTTP), 5μL [^{32}P]-dCTP (10μCi/μL), 26.5μL sterile water, 1μL nuclease free BSA (10mg/mL) and 2.5μL Klenow enzyme (2U/μL). The reagents were well mixed and incubated at room temperature for 1 hour for labelling to take place. After this time 1μL of the reaction was checked for incorporation of the label.

If the reaction was deemed to have worked, the labelling procedure was then stopped by the addition of 2.5μL 0.4M EDTA pH 8.0. The labelled probe was then purified from unincorporated nucleotides by sequential phenol/chloroform extraction and passing the aqueous extract (made up to 500μL with 150μL 20X SSPE and 300μL sterile water) through a NAP-5 column equilibrated with 6X SSPE. The probe was then eluted with 1mL 6X SSPE and 1μL counted (with 3mL scintillant) in a scintillation counter to ascertain the resultant activity of the probe. The probe was denatured before use by heating at 100°C for 10 minutes and was used in hybridisation as described in 2.2.3.16.3. Probes typically had a specific activity/μg template DNA of 1-2X10^9 cpm and were used at a concentration of ~1X10^6 cpm/mL.

Initially, probes were used in the manner described above. However, despite precautions, background was sometimes a problem on the blots, as can be seen in certain figures in Chapter 6. To resolve this, later probes were preadsorbed for 4 hours before use against a blank piece of nylon membrane in 20mL prehybridisation solution containing 200μL salmon sperm DNA as before.

2.2.3.17.2 Preparation of asymmetric PCR labelled probe

This method involved a modified PCR reaction similar to that described in 2.2.3.5. However, the concentration of the 'forward' primer was limited, so that in effect only the antisense strand was amplified, generating a single-stranded probe (this should be more sensitive than a conventional

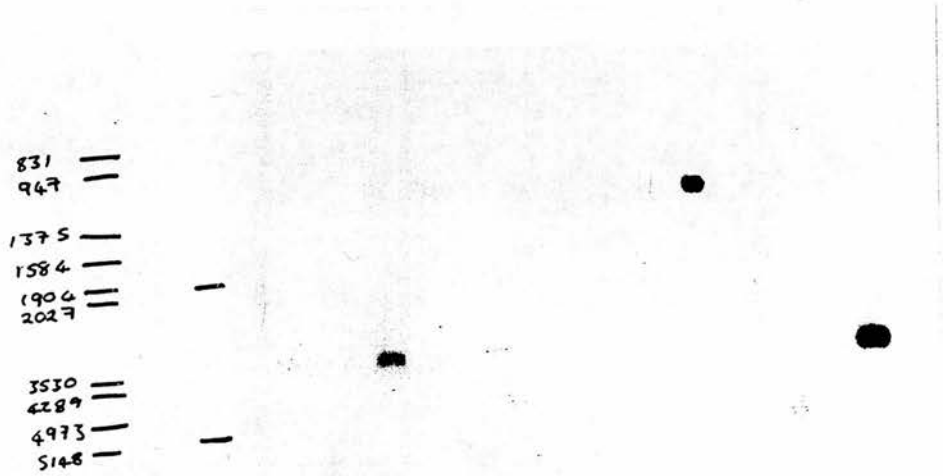


Figure 2.4

Autoradiograph of a Northern blot comparing the sensitivities of Vogelstein and asymmetric PCR AT_1 probes. 25µg bovine adrenal RNA was run in the lanes indicated, and with both probes, whether 1/6 or 5/6 of the probe was used, a 3.3kb species was detected. This corresponds to the size of the bovine adrenal AT_1 receptor cloned by Sasaki *et al.* 1991).The PCR probe is more sensitive, but the less sensitive Vogelstein probe also detects the AT_1 mRNA species. A 1.1kb species is also detected in the copy control lane, in which 25µg 3T3 fibroblast RNA was spiked with a small amount of AT_1 probe fragment. As little as 5pg AT_1 mRNA can be detected (copy 2). The sizes of the DNA and RNA markers are indicated alongside.

double-stranded probe as described in 2.2.3.17.1). The nucleotide composition also varied. α^{32}P-dCTP was used in excess, 50μCi/reaction, and dA,T and GTP were used at 2.5mM. A small amount of unlabelled dCTP was also included (0.25mM). The 'forward' primer concentration was 0.2μM, and the 'reverse' 20μM. 10ng AT_1 insert DNA was used as the template. All other conditions were as described in 2.2.3.5. After the PCR reaction, the product was checked for incorporation. If this was satisfactory, the probe was used directly, with no further purification. Immediately before use, it was denatured for 10 minutes at 100°C and chilled on ice.

Figure 2.4 shows an autoradiograph of a Northern blot of 25μg samples of bovine adrenal zfr cell RNA probed with both a Vogelstein probe and an asymmetric PCR probe to compare the sensitivity. Although the asymmetric probe was more sensitive, the Vogelstein probe detected the AT_1 mRNA at levels as low as 5pg (see copy controls), and therefore this simpler method of probe preparation was chosen.

2.2.3.18 Analysis of Northern blotting data

For qualitative analysis, the blots were exposed to Kodak X-ray film as described. For the quantitative analyses, however, the blots were laid down to Bio-rad BI phosphorimager screens and volume analysis of the resultant pixels in each band was performed, compensating for background in each case. This was done for both the AT_1 and β-actin - probed blots. The AT_1 bands were then normalised to the corresponding β-actin bands and the quantity of AT_1 receptor mRNA relative to that of the averaged basals was obtained. Statistical analysis of any significance in the changes observed was calculated using a paired t-test. Significance was inferred if a p-value of <0.05 was obtained.

Chapter 3 : Optimisation of a modified primary culture of bovine adrenal zona glomerulosa cells

3.1 Introduction

Primary cell cultures of the zona glomerulosa from the adrenal cortex have been used for many years as a model system to study the nature, function and regulation of angiotensin II receptors (Hornsby & O'Hare, 1977; Andoka *et al.* 1984; Douglas *et al.* 1984; Kojima *et al.* 1984), which mediate the stimulation of aldosterone secretion from these cells (Catt *et al.* 1979).These studies have focused on two model systems, each of which possesses specific advantages : the rat, because of its flexibility for conducting *in vivo* physiological and pharmacological investigations (Aguilera *et al.* 1980; Brilla *et al.* 1993); and the ox, because of its closer steroidogenic similarity to man (secreting cortisol as the principal glucocorticoid) and the availability of tissue (Neville & O'Hare, 1982).

Methods for isolation and culture of these cells have been widely published (Saltman *et al.* 1976; Crivello *et al.* 1982; Elliott *et al.* 1985; Shepherd *et al.* 1992). For bovine adrenal zona glomerulosa cultures, these largely rely on slicing the adrenal gland, digesting the subcapsular cells, and separating zg cells from other cell types by means of density gradient centrifugation. More variation is found in the culture conditions. Most investigators claim that to maintain the full steroidogenic capacity of the cells, certain antioxidant compounds are necessary in the culture medium. This was first proposed by Hornsby & Crivello (1983a), who suggested that the high natural antioxidant content of the adrenal gland was necessary to maintain steroid enzyme activity. The same group later demonstrated this to be the case (Crivello *et al.* 1982; Crivello *et al.* 1983; Hornsby & Crivello, 1983b), by showing that addition of metyrapone (a cytochrome P450 inhibitor) and

other antioxidants to the culture medium slowed or stopped the normal decline in steroid synthesising capacity of the cells.

The reason for the loss of steroid synthesising ability was believed to be the accumulation of basally-secreted steroids acting as pseudosubstrates for certain cytochrome P450s, especially 11β-hydroxylase. This was proposed to lead to uncoupling of the enzymes and generation of harmful lipid peroxides, which damage the cells (Crivello *et al.* 1983; Crivello *et al.* 1982; Hornsby, 1989). Uncoupling of the enzymes would effectively reduce the capacity of the cells to synthesise the appropriate steroids. The adrenal cortex is particularly high in lipids, rendering it especially vulnerable to such damage. To protect against this, it produces high levels of antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), glutathione, selenium and superoxide dismutase (SOD), a compound protectant against oxidative damage. These are therefore commonly included in the culture media of bovine adrenal zona glomerulosa cells (excepting SOD), with the addition of transferrin and insulin.

In this chapter, data are presented describing a modified method of bovine adrenal zona glomerulosa cell isolation, purification and primary culture. The main modification was in the purification step, where a Sephadex gel filtration system was used, and compared to, a more standard Percoll density gradient to purify the zona glomerulosa cells. Full details of both methods can be found in Chapter 2. The resulting cells, purified by column filtration, maintain their capacity to secrete aldosterone for up to six days in culture, without the use of antioxidants in the medium. Indeed, the use of antioxidants in this system appeared to inhibit steroidogenesis.

Using this modified culture procedure, the day-by-day responses of the cells to angiotensin II, and changes in ^{125}I-AII binding, were studied. This provided an insight into the functioning of the AII receptor during short-term culture. It also suggested that the observed decline in aldosterone synthesis was due not to a lack of steroid synthesising capacity, but to a decrease in AII receptor number in the cells.

3.2 Results

3.2.1 Purity of the culture

The purity of the slices used to isolate cells before Percoll or Sephadex purification was assessed by examination of formalin-fixed slices typical of those taken for the preparation. Slices were examined by light microscopy for cells exhibiting zona glomerulosa (high nuclear:cytoplasmic ratio; low numbers of lipid droplets) and zona fasciculata (lower nuclear:cytoplasmic ratio; higher numbers of lipid droplets) characteristics. The slices taken for the zg preparation were predominantly composed of cells with the morphological features of the zona glomerulosa (shown in Table 3.1). A light micrograph of a typical first slice from a bovine adrenal gland is shown in Figure 3.1, where the majority of the adrenocortical cells are zg cells, with a minority of zf cells on the inward face of the slice.

Purity of the cultures was also assessed by measuring the cortisol:aldosterone ratio for cells prepared using either Percoll gradient or Sephadex purification. If significant inner zone contamination were present, stimulation with AII or ACTH would be expected to produce larger amounts of cortisol compared to aldosterone secretion. All results for this and the following sections are triplicate determinations (plus/minus standard error of the mean) from representative experiments, which were performed at least twice. Where statistical significance has been determined, Student's t-test or one-way ANOVA has been used; significance was inferred when $p<0.05$.

The ratio of cortisol : aldosterone production was calculated for zona glomerulosa cells produced by both Percoll and column purification methods, as shown in Figure 3.2. A method similar to this for assessing culture purity was used by Braley *et al.* (1992), and also de Lean *et al.* (1984b). The data for aldosterone and cortisol secretion are also shown in Table 3.2. Bovine zg cells were cultured for four days after isolation. Upon stimulation with 10nM AII or 0.1nM ACTH, the cortisol:aldosterone ratio decreased

(when compared to basal), indicating a prevalence of aldosterone secretion in these cells, and consistent with a purified zona glomerulosa cell preparation with little or no inner zone cell contamination. There was no significant difference between the cortisol:aldosterone ratios for the two purification methods. Measurement of the ratio in further experiments gave a value of less than 10 in the basal state, suggestive of less than 35% contamination with zfr cells (de Lean *et al.* 1984b).

3.2.2 Comparison of two purification methods used in the culture

The Percoll gradient purification involved many centrifugation steps and forming the gradient was difficult. It was therefore felt that a modification of the purification method used in our laboratory for bovine zfr cells (Williams *et al.* 1989) might be an acceptable and more convenient alternative. The modified method of zona glomerulosa cell preparation using a Sephadex gel filtration protocol was then compared to the standard Percoll density gradient method.

Figure 3.3 shows a comparison of aldosterone secretion following stimulation with angiotensin II of zona glomerulosa cells prepared by either the Percoll or column purification methods. Cells were cultured for four days after isolation and stimulated with AII (10^{-12} - 10^{-7}M) for 3 hours. Aldosterone was measured in the overlying media. It can be seen that there is no significant difference between the two sets of cells. Figure 3.4 compares responses of the two cell preparations to maximal concentrations of AII, ACTH and K^+. Again, there is no significant difference between the two preparations for stimulation with AII and ACTH, while the column method gives better ($p<0.01$) results following stimulation with 12mM potassium.

Sample slice number	Zona glomerulosa (%)	Zona fasciculata (%)
1	60	40
2	80	20
3	60	40

Table 3.1

Table showing results of histopathological examination of outer slices from three sample preparations. These slices consist mostly of zona glomerulosa, with a minority of zona fasciculata cells and no zona reticularis. The mean proportion of zg in these slices was 67%, with standard error of 6.67%.

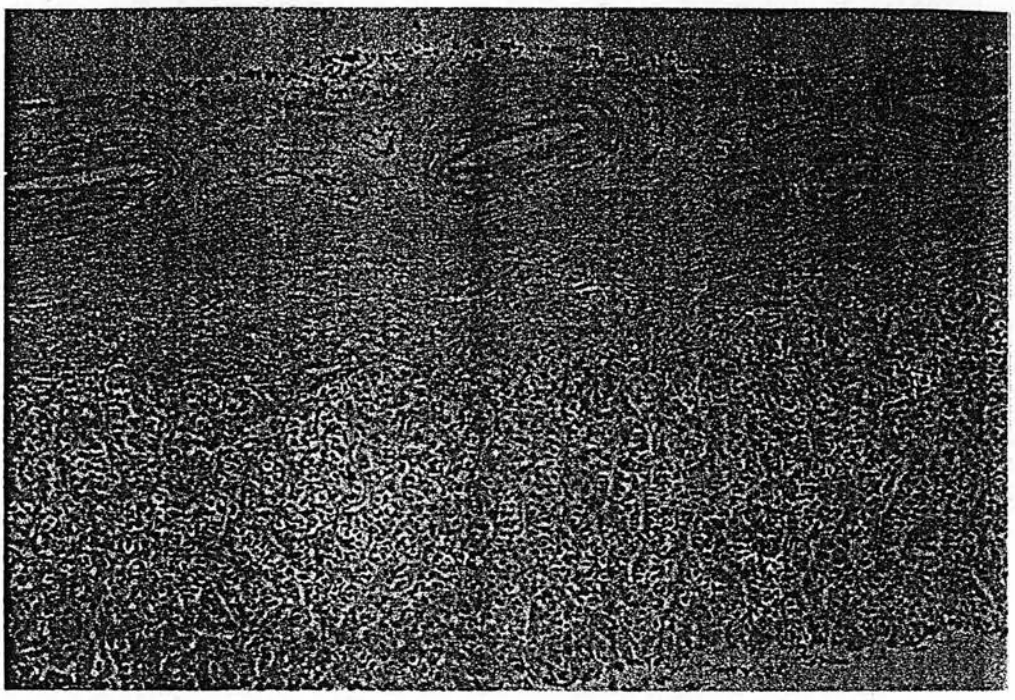


Figure 3.1

Haematoxylin/eosin-stained section of a typical outer slice from a bovine adrenal gland. Slices 100μm thick were cut from fat-trimmed glands and fixed in formalin. The slice shows the outer capsule, the entire zona glomerulosa and a small layer of inner zona fasciculata cells. Magnification is X200.

Stimulus	Aldosterone (Percoll)	Aldosterone (Sephadex)	Cortisol (Percoll)	Cortisol (Sephadex)
Basal	0.02 +/- 0.003	0.03 +/- 0.004	0.27 +/- 0.13	0.11 +/- 0.0
AII 10nM	0.22 +/- 0.01	0.26 +/- 0.03	0.32 +/- 0.06	0.16 +/- 0.02
ACTH 0.1nM	0.21 +/- 0.02	0.26 +/- 0.004	0.14 +/- 0.05	0.25 +/- 0.05
K^+ 12mM	0.08 +/- 0.01	0.22* +/- 0.03	0.35 +/- 0.15	0.12 +/- 0.05

Table 3.2

Steroid secretion rates (nM/mg protein) for bovine zg cells purified using Percoll or Sephadex. There is no significant difference between the two methods, with the exception of the potassium-stimulated aldosterone secretion, where the Sephadex-purified cells gave a greater response than the Percoll-purified cells (*$p<0.01$). This data is from a representative experiment which was performed three times.

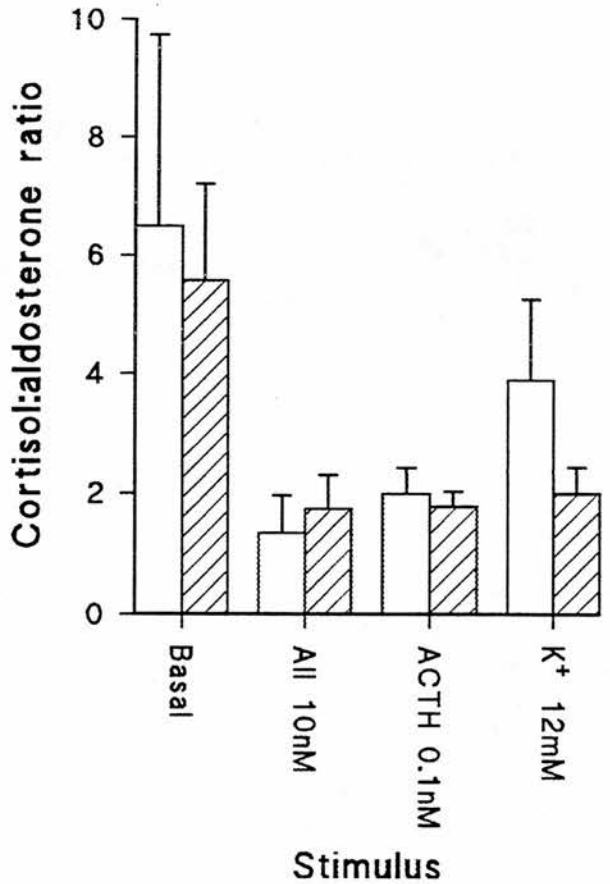


Figure 3.2

Ratios of cortisol : aldosterone secretion calculated for bovine zona glomerulosa cells purified by Percoll (□) or Sephadex column (▨). 20,000 cells/well were cultured for 4 days after isolation and stimulated for 3 hours with either 10nM AII, 0.1nM ACTH or 12mM K^+; data combined from three experiments. Stimulation with all three agonists decreased the ratio, indicating an increased production of aldosterone compared to cortisol. There was no significant difference in the cortisol:aldosterone ratio between the two sets of cells.

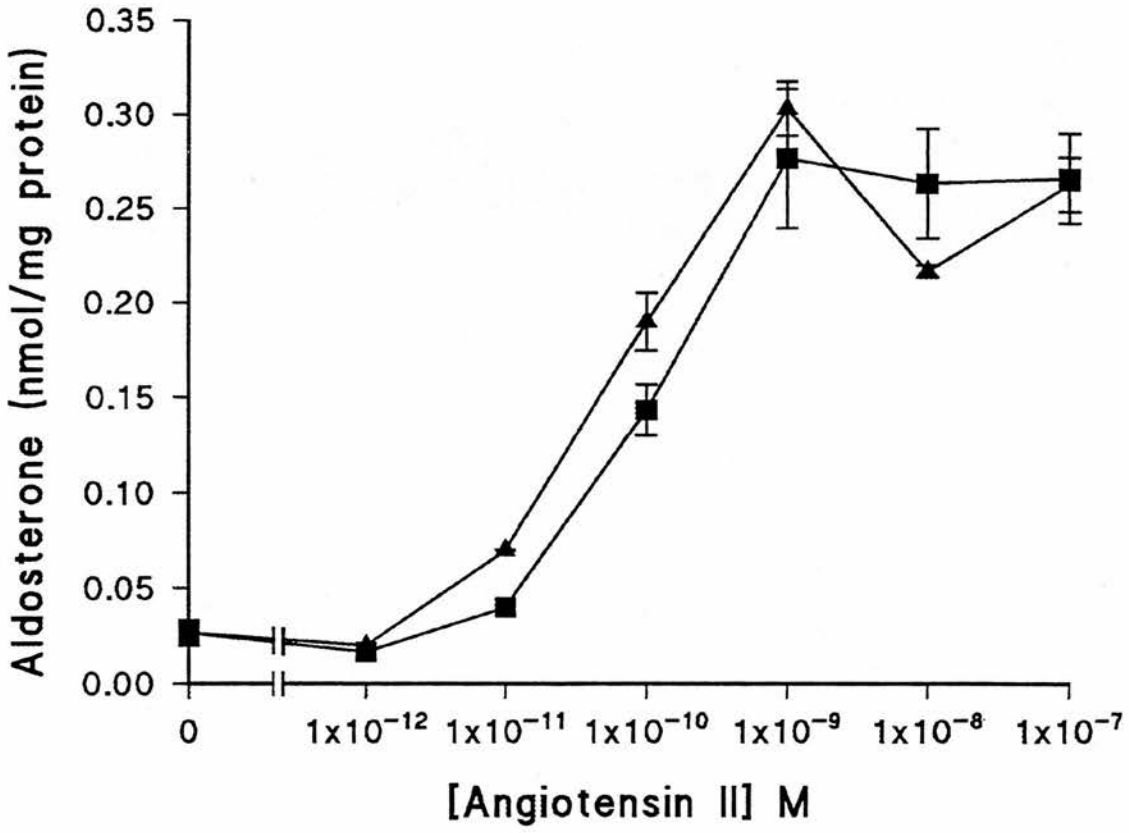


Figure 3.3

Dose-response curves to AII for bovine zg cells purified by Percoll (▲) or Sephadex (■). Bovine zg cells were prepared and isolated by the two methods, cultured for 4 days and stimulated with AII for 3 hours. The two sets of cells were taken from the same pool of glands. There was no significant difference between maximal aldosterone production induced by AII in the two preparations. Data taken from one representative experiment carried out on three separate occasions.

3.2.3 Changes in aldosterone response to AII stimulation during culture

The day-by-day aldosterone responses of cultured zona glomerulosa cells to a maximal AII stimulus of 10nM (compared to basal responses) for bovine zg cells from the day after isolation (day 1) to 5 days afterwards (day 6) is shown in Figure 3.5. These cells were prepared using the Sephadex column purification method. The maximum response was reached on day 5 of culture, n-fold stimulation reaching 6.88 (see Table 3.3). After this the response declines. Basal secretion, having remained fairly steady up until day 5, decreased on day 6. The aldosterone secretion of freshly isolated cells (day 0) was 0.348 +/- 0.03 nmol/mg protein basally, and 0.776 +/- 0.04nmol/mg protein following stimulation with 10nM AII.

3.2.4 Effect of antioxidants on aldosterone response to AII

As some of the antioxidants used in the culture medium were known to be inhibitory to steroidogenesis (e.g. metyrapone), the effect of the supplementary antioxidants in the medium on aldosterone secretion from the cells was investigated. In Figure 3.6 the aldosterone response of the cells to maximal concentrations of 10nM AII, 0.1nM ACTH or 12mM potassium (3 hours incubation) over days 1 to 6 in culture, for cells cultured with (Figure 3.6a) and without (Figure 3.6b) the antioxidant supplements in the medium are shown. Although basal responses were approximately equal for both culture conditions, the cells cultured without antioxidants showed a greater aldosterone response when compared to cells cultured with antioxidants. Differences were significant on day 3 for all agonists, on day 5 for ACTH and on day 4 for K^+ ($p<0.05$). Figure 3.7 shows a dose-response curve to AII for the two sets of cells carried out on day 5 of culture. The cells grown without antioxidants are significantly more sensitive ($p<0.05$) than those with reaching maximum response at 10^{-9}M AII. Cells cultured in the presence of supplementary antioxidants reached maximum response to AII at 10^{-8}M. A difference was also seen in the pattern of responsiveness after cell

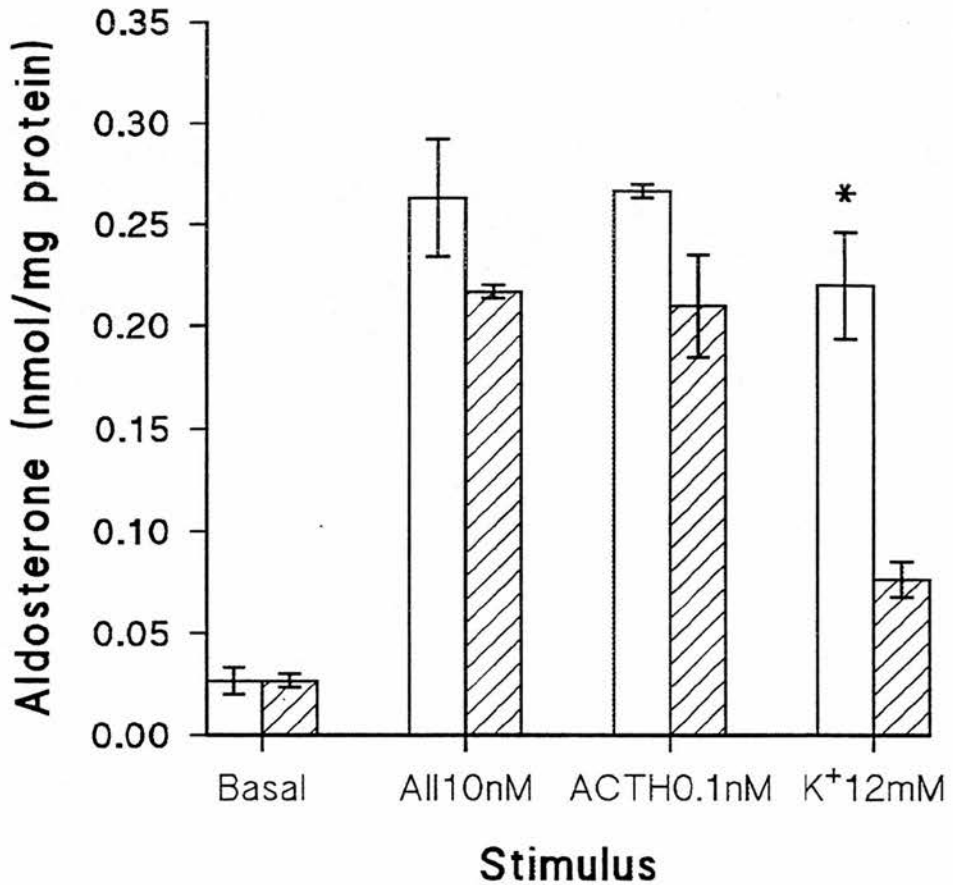


Figure 3.4

A comparison of aldosterone responses to maximal stimulatory concentrations of AII, ACTH and K^+ in bovine zg cells purified by Percoll (▨) or Sephadex (□). Cells were isolated from the same pool of adrenal glands and purified by either the Percoll density gradient or Sephadex column method, cultured for 4 days and then stimulated with 10nM AII, 0.1nM ACTH or 12mM K^+ for 3 hours. Cells purified on Sephadex produced slightly more aldosterone than the Percoll-purified cells after AII or ACTH treatment, and significantly more (* : $p<0.01$) after K^+ treatment. This data is from a representative experiment, which was performed three times.

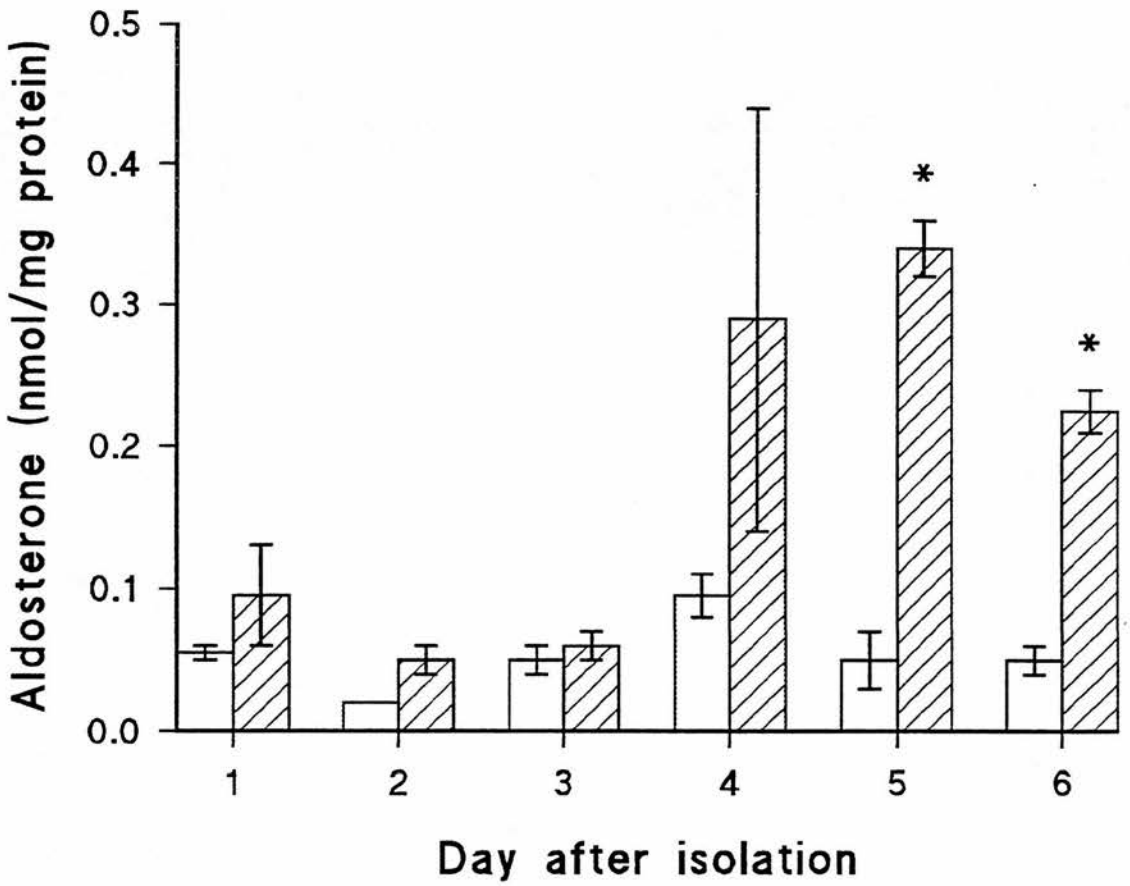


Figure 3.5

Day-by day aldosterone secretion of bovine zg cells purified using the Sephadex column method. The bars correspond to basal secretion (□) and secretion following stimulation with 10nM AII (▨) for 3 hours. These cells were cultured in the presence of antioxidants. Maximal response, corresponding to an n-fold increase over basal of 6.88, is reached on day 5 after isolation. * : $p<0.01$ compared to basal response; data from one of two representative experiments.

Figure 3.6

Day-by-day aldosterone responses for 3 hour exposure to a maximal stimulus of 10nM AII (▨), 0.1nM ACTH (▩) and 12mM K^+ (▧). Basal aldosterone secretion (□) is also shown. Both panels show bovine zg cells isolated from the same pool of glands, purified by Sephadex gel filtration, and cultured in the presence (Figure 3.6a : upper panel) or absence (Figure 3.6b : lower panel) of supplementary antioxidants. In the absence of antioxidants, responses in all cases were higher, significantly so on days 3-4 (AII and K^+) and 3-5 (ACTH); basal secretion was also greater on day 4 ($p<0.05$). The day of maximal response was later (day 5) in the presence of antioxidants than in their absence (day 4). Data taken from one of two representative experiments. (** $p<0.01$; * $p<0.05$ indicates responses significantly greater than basal).

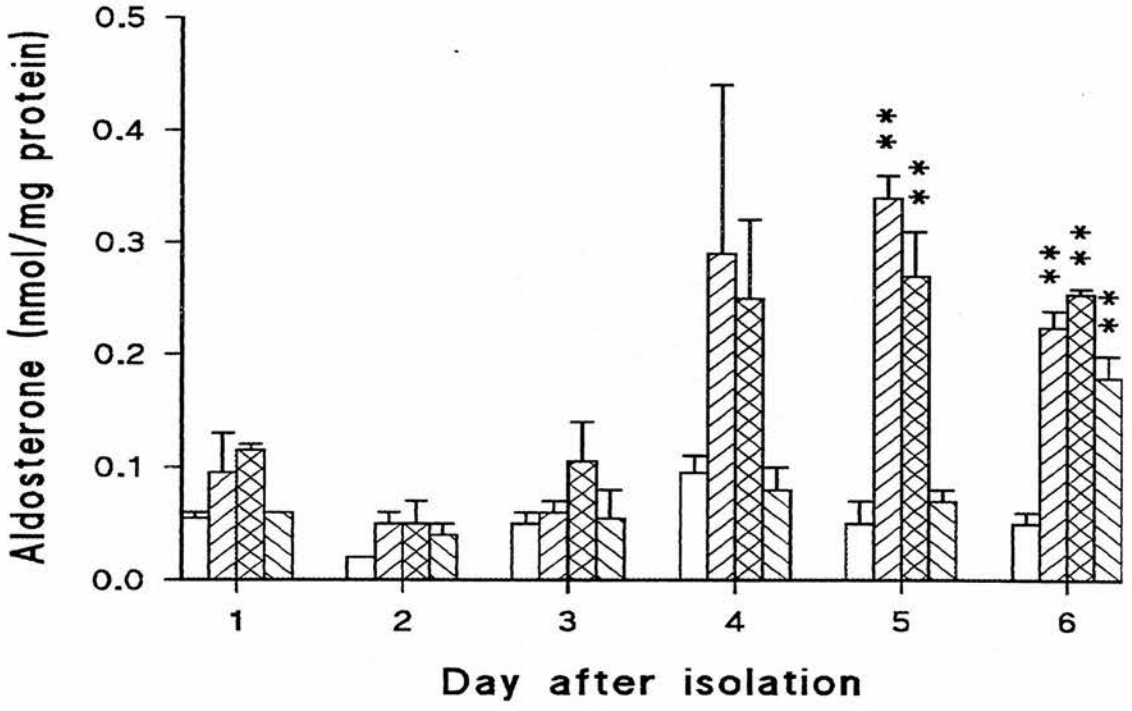


Figure 3.6a

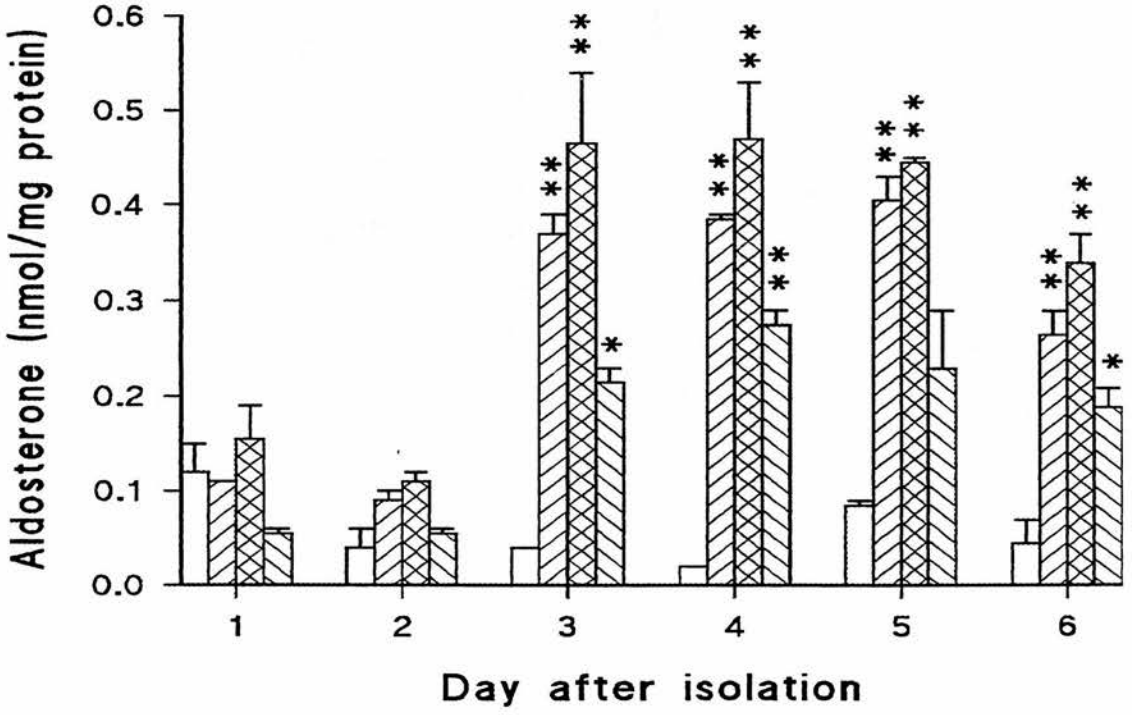


Figure 3.6b

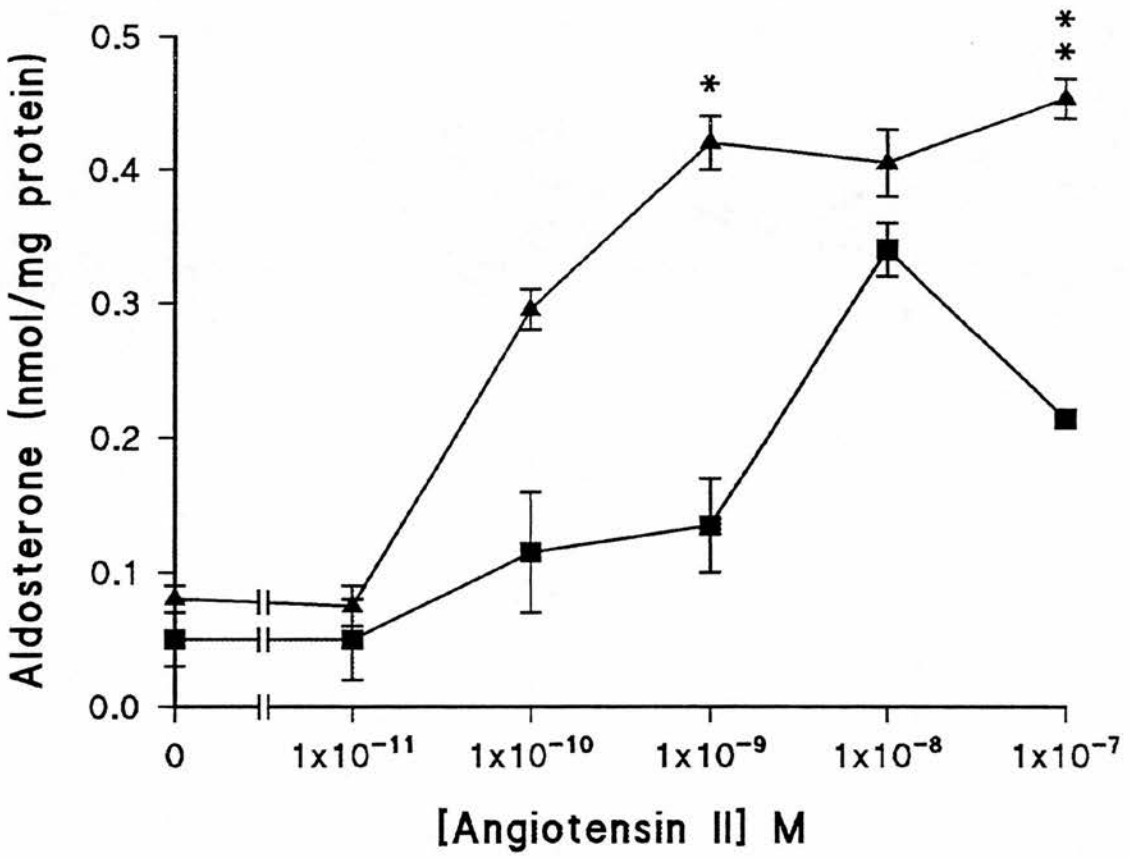


Figure 3.7

Dose response curve to AII for bovine zg cells in the presence (■) or absence (▲) of antioxidants in the culture medium. Cells were cultured for 5 days after isolation in each case; both sets of cells were taken from the same preparation. The cells without antioxidants appeared to be more sensitive to AII, reaching a maximum aldosterone response at 10^{-9} M AII. In contrast, those cultured with antioxidants appeared less sensitive, reaching a maximum at 10^{-8}M AII. ** : $p<0.005$; * : $p<0.05$ for greater response in the absence of antioxidants. Data from a representative of two similar experiments.

isolation for the two sets of cells to AII : n-fold stimulation (over basal) for cells without antioxidants was maximum on day 4 as compared to day 5 for cells with antioxidants. This can be seen in Table 3.3.

A comparison of basal and AII-stimulated aldosterone secretion in bovine zg cells, prepared using Sephadex purification and cultured without antioxidants, with that in bovine zg cells published by two other groups (de Lean *et al.* 1984b; Shepherd *et al.* 1992) is given in Table 3.4. Although basal secretion is less than in Shepherd *et al.* (1992), AII-stimulated aldosterone secretion was greater than in both published datasets.

3.2.5 Changes in AII binding (receptor number) during culture

These studies were of a preliminary nature to examine the relationship between maximal AII binding and maximal aldosterone responsiveness to AII. Data from day-by-day radioligand binding studies (using ^{125}I-AII) on zona glomerulosa cells in culture are shown in Figure 3.8. Maximal ^{125}I-AII binding was reached on day 4 in culture (parallel to the steroid secretion data) and fell to a plateau thereafter. These data were, however, uncorrected for cell number and when this was done (by correcting for protein samples taken on days 3 and 6) it appeared that until day 4 the cells were growing and expressing functional receptors; after day 4 the cells continued to grow and appear healthy but were possibly unable to express functional AII receptors and therefore AII binding decreased. The actual receptor number and affinity (B_{max} and K_D) were not calculated for each day, but were calculated for the day showing maximal ^{125}I-AII binding. The results were as follows : $K_D = 3.9 \times 10^{-10}$M (range 2.0-6.8 $\times 10^{-10}$M); B_{max} = 566.8fmol/10^6 cells (range 190-943.5 fmol/10^6 cells).

Day after isolation	Aldosterone (n-fold increase over basal) *with antioxidants*	Aldosterone (n-fold increase over basal) *without antioxidants*
1	1.7 +/- 0.49	1 +/- 0.24
2	2.5 +/- 0.5	2.3 +/- 1.17
3	1.2 +/- 0.04	9.3 +/- 0.5 *
4	3.1 +/- 1.13	19.5 +/- 0.25 *
5	6.8 +/- 2.77	4.6 +/- 0.02
6	4.6 +/- 0.63	6.8 +/- 5.54

Table 3.3

This table shows the n-fold increases over basal aldosterone secretion from bovine zona glomerulosa cells stimulated with 10nM AII for 3 hours, on various days after isolation. Cells were cultured in the presence or absence of supplementary antioxidants as described in Chapter 2. Note that the cells are more sensitive, having a greater aldosterone response in the absence of antioxidants which was statistically significant (* : $p<0.005$) on days 3 and 4 after isolation. Data drawn from one of two representative experiments.

bovine zg cells	Basal	AII-stimulated
experimental	2.33 +/- 0.3*	39.08 +/- 4.2**
(Shepherd *et al.* 1992)	5.49 +/- 0.59	19.23 +/- 0.9
(de Lean *et al.* 1984b)	1.11 +/- 0.24	6.58 +/- 1.90

Table 3.4

Basal and AII-stimulated aldosterone secretion rates (on d4 after isolation), expressed as pmol/10^6 cells/3h (mean +/- s.e.m), for bovine zg cells purified by Sephadex column filtration and cultured in the absence of antioxidants. Similar rates for two published methods are given for comparison. Basal secretion in our hands was significantly less (*$p<0.001$) than that of Shepherd *et al.* (1992); however, AII-stimulated secretion was significantly greater than in both published methods (**$p<0.001$). This represents the combined data from four experiments.

3.2.6 Characterisation of the AII receptor subtypes in bovine adrenal zona glomerulosa cells

We wished to confirm whether the ^{125}I-AII binding observed was, as previously documented (Balla *et al.* 1991; Ambroz & Catt, 1992; Rainey *et al.* 1991), to the AT_1 receptor subtype. Bovine zg cells were cultured without antioxidants for four days after isolation. They were then incubated for 2.5 hours with ^{125}I-AII in the presence or absence of unlabelled AII, DuP753 or PD123177. Figure 3.9 shows that ^{125}I-AII binding was almost completely displaceable by the AT_1-selective antagonist DuPont 753. A small (13%) but significant ($p<0.05$) proportion of the binding was displaced by the AT_2-selective compound PD123177. This confirmed that the AII receptor population in the culture was predominantly AT_1 in nature.

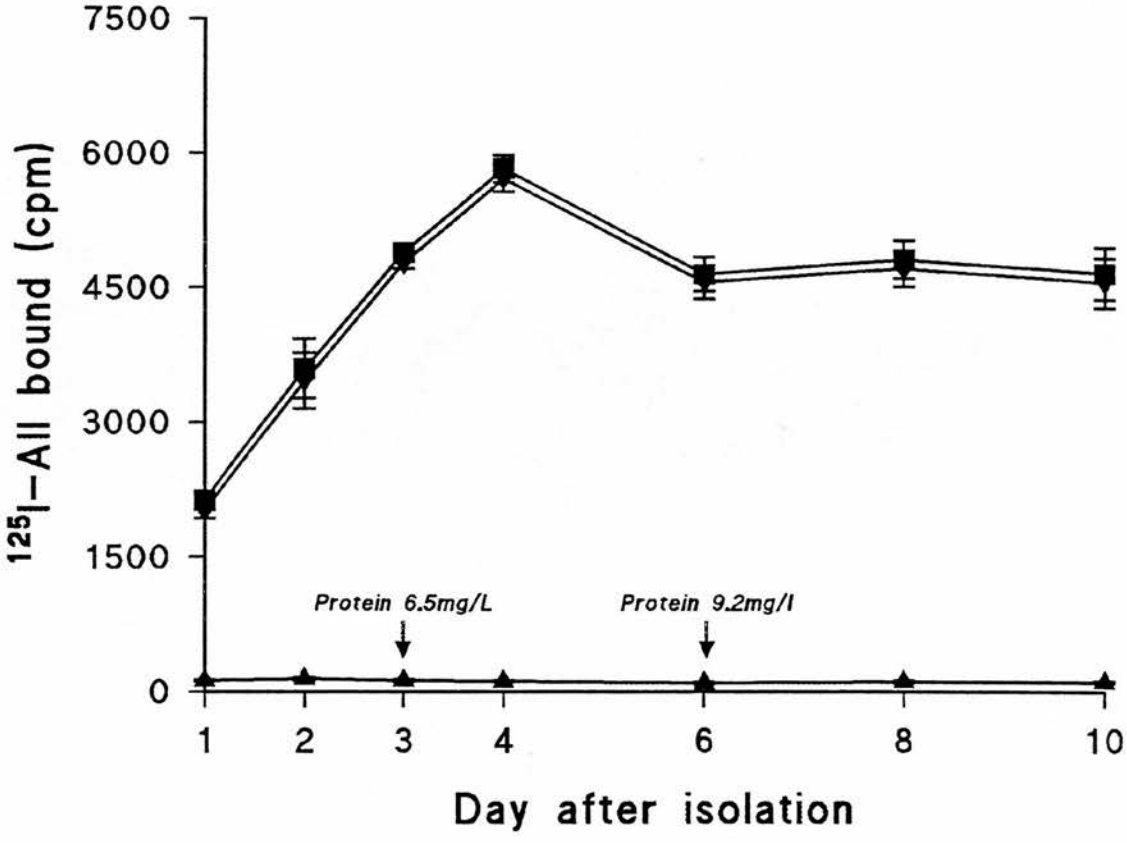


Figure 3.8

Changes in the amount of ^{125}I-AII bound by cultured bovine zona glomerulosa cells on different days in culture. The binding increased sharply up until day 4 after isolation, after which it was reduced to a plateau. This gave an approximate measure of AII receptor number, which appeared to reach a maximum on day 4, similar to the aldosterone response. The maximum receptor concentration, B_{max}, was calculated for day 4 and was 943.5fmol/10^6 cells. This graph also shows how the protein in each well changed during culture : an approximation of cell number. The well protein content increased throughout culture. (■ = total, ▲ = non-specific, and ▼ = specific ^{125}I-AII binding). These data were taken from one of two representative experiments.

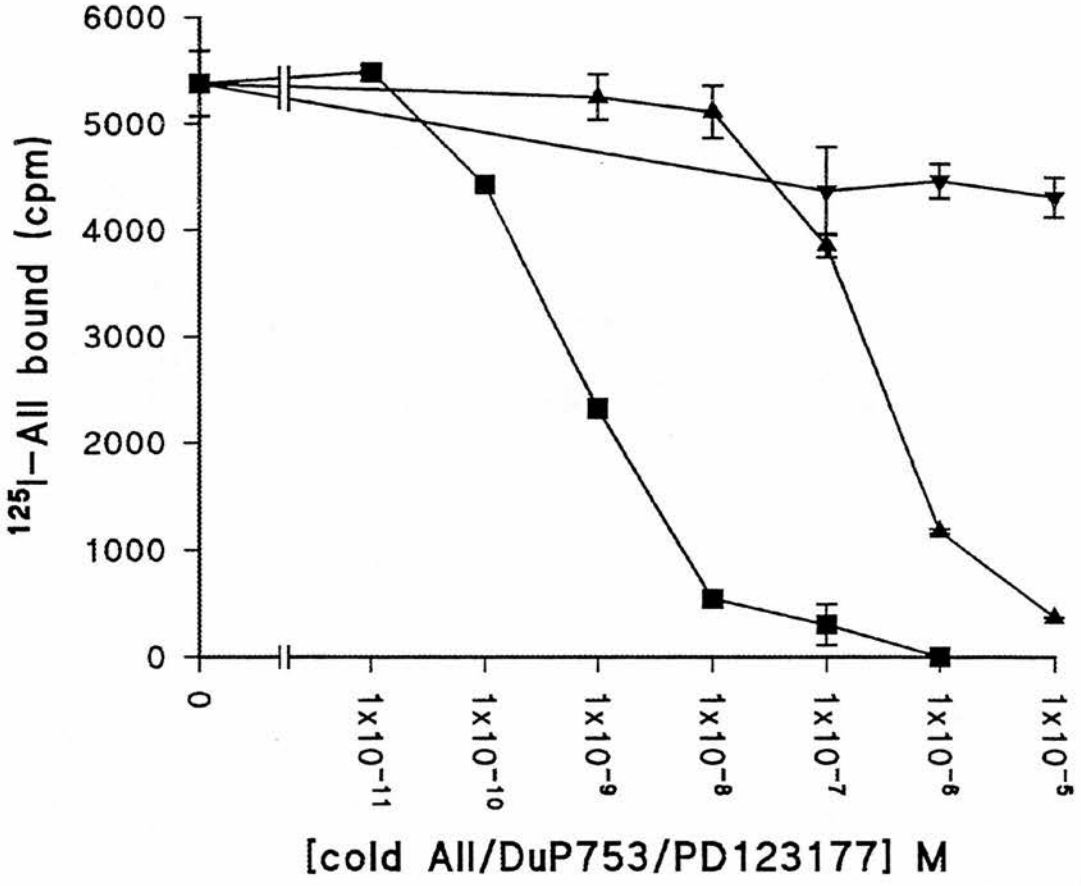


Figure 3.9

Displacement of ^{125}I-AII from day 4 cultures of bovine zg cells by ■ unlabelled AII, ▲ DuPont 753, or ▼ PD123177. AII displaced ^{125}I-AII with an ID_{50} of 6.8×10^{-10}M, allowing a calculation of the receptor affinity (K_D) to be made at 6.3×10^{-10}M. DuPont 753 displaced almost all the ^{125}I-AII, with an ID_{50} of 3.3×10^{-7}M, while PD123177 displaced a small but significant ($p<0.05$) proportion of the ^{125}I-AII. Data taken from a representative of two experiments.

3.3 Discussion

Previously published methods for culture of bovine zg cells (Shepherd *et al.* 1992; Gyurko *et al.* 1992) were modified by using an alternative method for purifying the cells after digestion. The Sephadex column modification, adapted from that used to purify bovine zfr cells (Williams *et al.* 1989) was thought to be quicker and less damaging to the cells than the Percoll method, due to the decreased number of centrifugation steps. To test this hypothesis, it was necessary to compare the purity and responsiveness to steroidogenic stimuli (AII, ACTH, K^+) of the two preparations.

Both isolation and purification methods used in these studies routinely produced viable cultures of zona glomerulosa cells, with low contamination by zona fasciculata/reticularis cells. This was confirmed in two ways.

Firstly, light microscopy analysis of formalin-fixed slices representative of those used for preparation of zg cells indicated the majority of the cells (60-80%) to be zg cells, as (Table 3.1). The photograph of a section of a typical outer slice (Figure 3.1) also illustrates that only a small proportion of the cells showed zf characteristics (lower nuclear:cytoplasmic ratio and higher numbers of lipid droplets than zg cells). The accuracy of the slicing technique used was therefore crucial to achieving a preparation where zf contamination was kept to a minimum. Both Percoll density gradient and Sephadex column purification methods removed red blood cells and cellular debris.

Secondly, levels of zf contamination were assessed by calculating the cortisol : aldosterone ratio as (Figure 3.1). According to de Lean *et al.* (1984b), a ratio of less than ten indicates zf contamination to be less than 35%. All ratios obtained in this comparison, and continuing monitoring of the cells, were consistently below this level. The cells purified on Percoll had a slightly higher basal ratio than those purified on the column. However,

stimulation with AII and ACTH gave ratios which were almost identical. The reason for the higher basal production of cortisol* in Percoll-purified cells (Table 3.2) is unknown; it is unlikely that the Percoll could have had any lasting effect on the cells to lead to this result as great care was taken to wash any Percoll residue away from the cells before plating. This comparison indicates that the Sephadex method was as effective as Percoll at purifying zg cells from red blood cells and debris, and that no preferential loss of zg cells was being observed during the gel filtration process.

The Sephadex column gel filtration method yielded cells with equivalent responses to steroidogenic stimuli to the Percoll-purified cells, as seen in Figure 3.3. The Sephadex-purified cells had, however, a significantly better response to potassium than cells purified on the Percoll density gradient. The reason for this is unknown; it could be due to a difference in purification but since the cells had been in culture 5 days before stimulation, this is unlikely. In the absence of any significant difference between cells produced by the two methods, the Sephadex purification was chosen for further use due to its increased speed and simplicity.

When stimulated with angiotensin II, ACTH and K^+, zona glomerulosa cells purified by Sephadex filtration produced increased amounts of aldosterone. Figure 3.5 shows stimulated aldosterone secretion data expressed on a daily basis, from day 1 until day 6 of culture. For the first three days after culture, there was no significant increase in aldosterone production following stimulation with 10nM AII, (the increase was significant in freshly isolated cells). Aldosterone secretion appeared to rise for the next two days reaching significance over basal on days 5 and 6. A maximal response was observed on day 5 after isolation.

For some time our laboratory has been successfully culturing bovine inner zone adrenocortical cells (Walker *et al.* 1988; Williams *et al.* 1989) without the use of antioxidants to maintain steroidogenic response. The postulated protective effect of such supplements (Crivello *et al.* 1982) routinely used by many investigators (Andoka *et al.* 1984; Balla *et al.* 1991;

* and lack of stimulation by any of the agonists used

Ambroz & Catt, 1992; Shepherd *et al.* 1992; Shizuta *et al.* 1992) was therefore investigated by culturing cells from the same preparation in medium with or without the supplementary antioxidants and growth factors. The cells were stimulated with maximal doses of AII, ACTH and potassium, and aldosterone responses in the two sets of media were compared. Without antioxidant supplements the cells were able to secrete aldosterone both basally and in response to steroidogenic agonists over the subsequent days. By days 3-5 after culture, a significant level of stimulated aldosterone secretion was observed (see Figure 3.6 and Table 3.3). This observation is in direct contradiction with that of Hornsby's group (Crivello *et al.* 1982; Crivello *et al.* 1983) who were unable to detect aldosterone synthesis upon stimulation with AII after 4 days' culture in the absence of antioxidants. The capacity of the zg cells in our hands to synthesise aldosterone from acute stimuli seems to be actually diminished by the addition of antioxidant supplements to the growth medium. On days 4 and 5 after isolation, the n-fold increase over basal in aldosterone response to AII is significantly less in the presence of antioxidants than in their absence (Table 3.3)

Experiments to determine which of the supplements was responsible for this were not undertaken. It is possible that metyrapone, which is a known reversible inhibitor of cytochrome P450 (Crivello *et al.* 1982) exerted some effect on the cells which was not reversed after the medium containing the drug had been thoroughly washed away prior to stimulation. Other investigators (Balla *et al.* 1991; Ambroz & Catt, 1992) incubated the cells in antioxidant-free medium for one hour before commencing experiments. This may have been a more effective method for removal of metyrapone. An alternative explanation is that the serum substitute used in this laboratory (CPSR-1) contained a different balance of antioxidants than the typically-used FCS, and that this allowed the cells to regain aldosterone synthetic capacity in their absence. If a different antioxidant balance was present in the serum substitute, it is possible that a toxic excess may then have been established by the addition of more antioxidants. Recently, Gallo-Payet *et al.*

(1993) showed a similar pattern to that reported here, but for cultures of rat zona glomerulosa cells : no antioxidants were used for 5 days of culture, with no significant loss of aldosterone synthesis (similar to the results in de Lean *et al.* (1984b) in bovine adrenal subcapsular cells) and it was concluded that the cells did not need antioxidants in such short-term cultures. This group also demonstrated an increasing sensitivity of the cells to stimulation with AII and ACTH, with falling basal levels of steroid production and increased stimulated levels; our day-by-day results also showed this effect (see Table 3.3).

Hornsby's group have previously published data showing that accumulation of steroids in culture media can inhibit further steroid production (Crivello *et al.* 1982; Crivello *et al.* 1983; Hornsby, 1989), and that this can be reversed by inclusion of antioxidants and other protectant compounds in the culture medium. The data from my studies indicated that, in the bovine adrenal zona glomerulosa culture described, these effects are not sufficiently pronounced to necessitate supplementing the medium. Indeed, basal aldosterone secretion in our cultures using no antioxidants showed significantly lower values than another group who used these antioxidants ((Shepherd *et al.* 1992); see Table 3.4). However, stimulation with AII led to a significantly greater aldosterone secretory response in cultures grown in the absence of antioxidants. It may be that for some reason the Sephadex purification method yields cells which are naturally more rich in antioxidants such that they resist depletion under basal conditions for the first few days in culture. This could account for our observations that cells grown in unsupplemented medium regain their steroidogenic capacity and also, perhaps, the inhibitory affect of these supplements : possibly antioxidant "overdose" is occurring (e.g. metyrapone, when not acting as an antioxidant, may inhibit conversion of deoxycorticosterone to corticosterone).

Aldosterone secretion in zg cultures without antioxidants reached a maximum (on stimulation with 10mM AII) after 4 days in culture,

decreasing after this. Radioligand binding studies using ^{125}I-AII gave concordant results, with maximum binding seen on day 4 (see Figure 3.7). However, protein data taken from sample wells after completion of binding experiments indicated that the cells were still growing or increasing in number after day 4. Thus the cells after 4-5 days in culture appear to lose their AII receptors, which would explain the decrease in steroidogenic response to AII after day 5. Gallo-Payet *et al.* (1993) have also demonstrated a loss of AII receptors during culture in rat zg cells. Thus although bovine zona glomerulosa cells can be maintained in culture without supplementary antioxidants for at least 6 days and remain healthy, during this time they lose their receptors for AII. It is also possible that AII receptor affinity is decreasing with time in culture. The results also suggest that the loss of steroidogenic capacity after the first few days in culture is not related directly to unavailability of the AII receptor but to some intracellular uncoupling of the receptor leading to an inability to produce steroids.

The radioligand binding data also showed that binding of ^{125}I-AII in bovine zg cells could be displaced by DuPont 753 and also, but to a much smaller extent by PD123177 (Figure 3.8), confirming that the majority of the receptors expressed in the cells on day 4 were of the AT_1 subtype. This observation is in agreement with that of other investigators, including Ouali *et al.* (1992), who found a small (15%) proportion of AT_2 receptors in bovine adrenocortical cells. It also agrees with the observation of Clyne *et al.* (1993) for cultures of bovine adrenal zona fasciculata/reticularis cells.

In conclusion, this chapter reports a modification to established methods for bovine adrenal zona glomerulosa cell culture which allows easier and faster cell isolation. The zg culture does not require antioxidants to maintain steroidogenesis; indeed, antioxidants seem to depress steroidogenesis. The changes that occur in zona glomerulosa cell responses to stimuli during continued culture are also reported. Aldosterone secretion (in response to AII) remains low for 2-3 days then rises, with a parallel

increase in apparent AII receptor number. Subsequently, steroidogenic response and AII binding decreases after 4-6 days in culture, probably due to loss of expression of the receptor. What leads to this loss of AII receptor binding is not clear. We have confirmed the findings of other researchers (Balla *et al.* 1991; Ouali *et al.* 1992) that the majority of AII receptors present in the culture are of the AT_1 subtype.

Chapter 4 : Pharmacological characterisation of AT_1 receptors in bovine zona glomerulosa and rat mesenteric vascular smooth muscle.

4.1 Introduction

The wide range of functions mediated by angiotensin II (discussed in Chapter 1) are consistent with the known heterogeneity within AII receptors. Following the advent of non-peptide AII antagonists, AII receptors were divided into types 1 and 2, with the known physiological effects being mediated by the type 1 receptor. In rodents, the (AT_1) receptors in the adrenal cortex and vascular smooth muscle were known to be regulated in opposite directions, (Aguilera & Catt, 1981, see 1.4.5.1) in response to changes in dietary Na^+. Before the subdivision of rodent AT_1 receptors into types AT_{1a} and AT_{1b}, as a result of molecular cloning studies, it was thought that some pharmacological difference between the two receptor subtypes might underlie this difference in regulation.

This hypothesis was strengthened when Clyne *et al.* (1993), using Schild analysis to obtain pA_2 values, found that the AT_1 receptor present in cultured bovine adrenal zona fasciculata/reticularis cells appeared to be pharmacologically distinct from that in rabbit aortic vascular smooth muscle (Chiu *et al.* 1990) (see Table 4.1).

Measurement of antagonist pA_2 values was first proposed as a method of classifying antagonists by Schild (Schild, 1947; Simonian & Gill, 1979), and the model discussed by Arunlakshana and Schild (1959) is now widely accepted as a means of receptor classification. The pA_2 value, defined as the negative logarithm of the concentration of an antagonist required to shift the dose-response curve of an agonist twofold to the right, is unique for a specific receptor. Thus, the pA_2 value for a particular agonist-receptor-antagonist combination should be constant wherever the location of the

receptor (Arunlakshana & Schild, 1959; Mackay, 1978). Conversely, a difference in pA_2 values is highly indicative of the presence of different receptor subtypes (Kenakin, 1987).

To clarify the discrepancy observed in pA_2 values for DuP753 at AT_1 receptors (Clyne *et al.* 1993), I report here the characterisation of the AT_1 receptors in two types of tissues. The systems used for this comparison were two cell culture preparations : bovine adrenal zona glomerulosa cells and rat mesenteric artery smooth muscle cells. Although the pA_2 value obtained by Schild regression analysis for a given competitive antagonist binding to the same receptor should not vary greatly between species (Kenakin, 1987), it was intended initially to compare the two different tissue types within the same species. The analysis in vascular tissue was thus firstly attempted in cultured bovine pulmonary artery smooth muscle cells. Unfortunately, no AII receptors were expressed in these cells and so a model of rat vascular smooth muscle cells, known to express functional AT_1 receptors (Lyall *et al.* 1992), was utilised instead. The responses to AII used in the analyses were aldosterone secretion from bovine zg cells, and phosphatidylinositol turnover in rat mesenteric artery vsmc. The antagonist used was the AT_1-selective competitive non-peptide antagonist DuPont 753.

The aims of this chapter were to perform Schild regression analyses using bovine zona glomerulosa and rat mesenteric artery smooth muscle cells in culture and from these to obtain pA_2 values for DuP753. These values were then compared with each other and previously published pA_2 values (Chiu *et al.* 1990; Clyne *et al.* 1993) in order to determine whether the AT_1 receptors analysed were pharmacologically distinct.

4.1.1 Theory of Schild regression analysis

Schild regression analysis relies upon the premise that to obtain an identical effect in the presence or absence of an antagonist, the same number of receptors must be being activated. The dose-response curve to an agonist

will therefore be shifted to the right in a parallel fashion when increasing amounts of a competitive antagonist are added to the incubations.

If the antagonist used is truly competitive, and produces a parallel rightwards shift in the agonist dose-response curves, Schild regression analysis can be performed. The ratio of agonist concentration producing a half-maximal response in the presence and absence of an antagonist conforms to the following equation, known as the Schild equation :

$$\log (dr - 1) = \log [B] - \log K_B$$

where dr is the dose ratio, [B] is the concentration of antagonist, and K_B is the equilibrium dissociation constant for the antagonist.

The logarithm of the dose ratio minus 1 can be plotted against the negative logarithm of the antagonist concentration to produce a regression line. If the regression is linear and of slope not significantly different from unity, the intercept of this line on log [B] yields the K_B. When the dose ratio equals two, $pK_B = - \log (dr - 1)$. Therefore, the pK_B equals the pA_2 when the slope of the regression is equal to unity. The intercept on the abscissa (at $y=0$) thus always yields the pA_2 for the antagonist, but the K_B only when the slope is equal to unity.

4.2 Results

The results shown in this section were obtained from triplicate determinations from representative experiments which were performed at least three times. Data from cumulative cell preparations were used in the Schild regression analyses. Dose-response curves are plotted as the mean +/- s.e.m and fitted to a log dose-response curve by the curve-fit package of FigP.

4.2.1 Dose-response curves in zg cells

Bovine zg cells were cultured as detailed in 2.2.1.1, and plated into 96-well plates at 20,000 cells/well. Aldosterone secretory responses to a range of concentrations of AII (AII dose-response curves) were measured on day 4 after isolation, as in 2.2.2.1, in the absence and presence of DuP753 at 3.3×10^{-7}, 1×10^{-6} and 3.3×10^{-6}M. DuP753 was added to the cells 5min before AII in order to establish binding between the antagonist and the receptor. The cells were stimulated with AII for 3h after which aldosterone secretion into the overlying medium was measured by RIA. These concentrations of DuP753 were used after reference to both previous work on Schild analysis of AT_1 receptors in bovine zfr cells (Clyne *et al.* 1993), and displacement of ^{125}I-AII from bovine zg cells by DuP753, which yielded an IC_{50} of 3.3×10^{-7} (Figure 4.1). Concentrations of DuP753 up to one log unit either side of the IC_{50} were tested for their ability to displace AII dose-response curves; the three concentrations giving consistent displacements were chosen (3.3×10^{-7}, 1×10^{-6} and 3.3×10^{-6}M DuP753). Figure 4.2 shows dose response curves to AII shifted to the right in a parallel fashion with increasing concentrations of DuP753, without the maximal response being lowered.

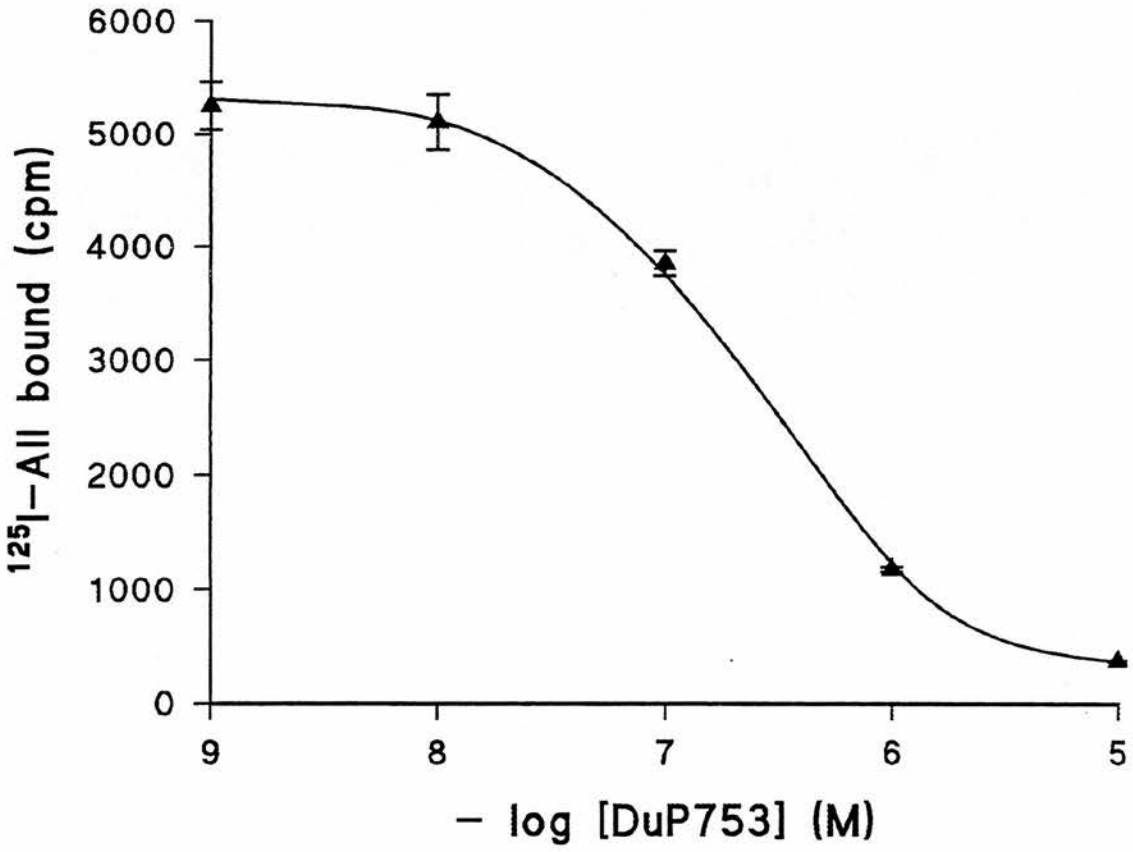


Figure 4.1

Determination of IC_{50} for DuP753 in bovine adrenal zg cells. Increasing concentrations of DuP753 were used to displace ^{125}I-AII from AT_1 receptors in cultured zg cells. Cells were cultured for 4 days after isolation and incubated with 100,000 cpm (0.05pmol) ^{125}I-AII in the presence of DuP753 at the concentrations shown for 2.5 hours. Displacement of binding to 50% of maximum was observed at 3.3×10^{-7} M DuP753. Representative data from one of two experiments.

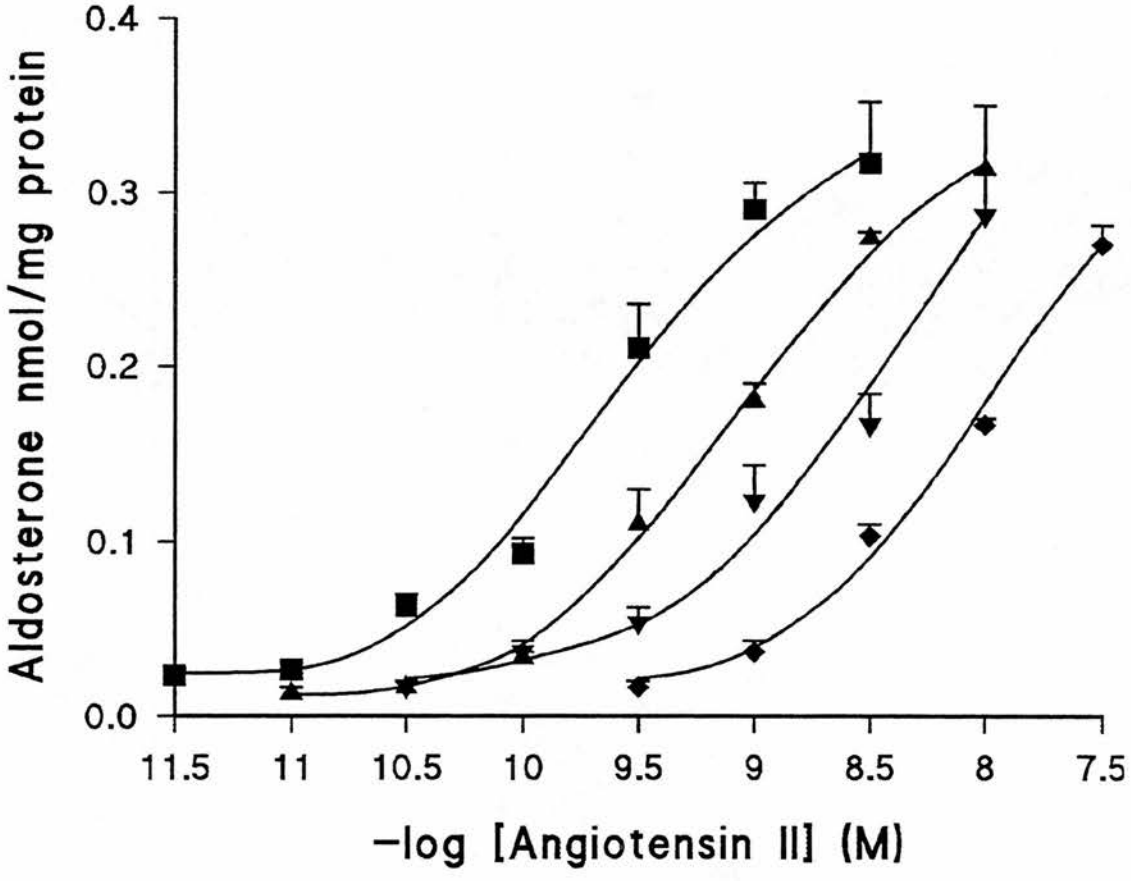


Figure 4.2

Displacement of dose-response curves to AII by increasing concentrations of DuP753. Bovine zg cells were cultured for 4 days after isolation and then stimulated with a range of concentrations of AII for 3 hours. Aldosterone secretion was measured in the absence (■) or presence of 3.3×10^{-7} M DuP753 (▲), 1×10^{-6} M DuP753 (▼) or 3.3×10^{-6} M DuP753 (◆). The curves shift to the right in a parallel fashion with no significant depression in the maximal response. Data from a representative experiment repeated three times.

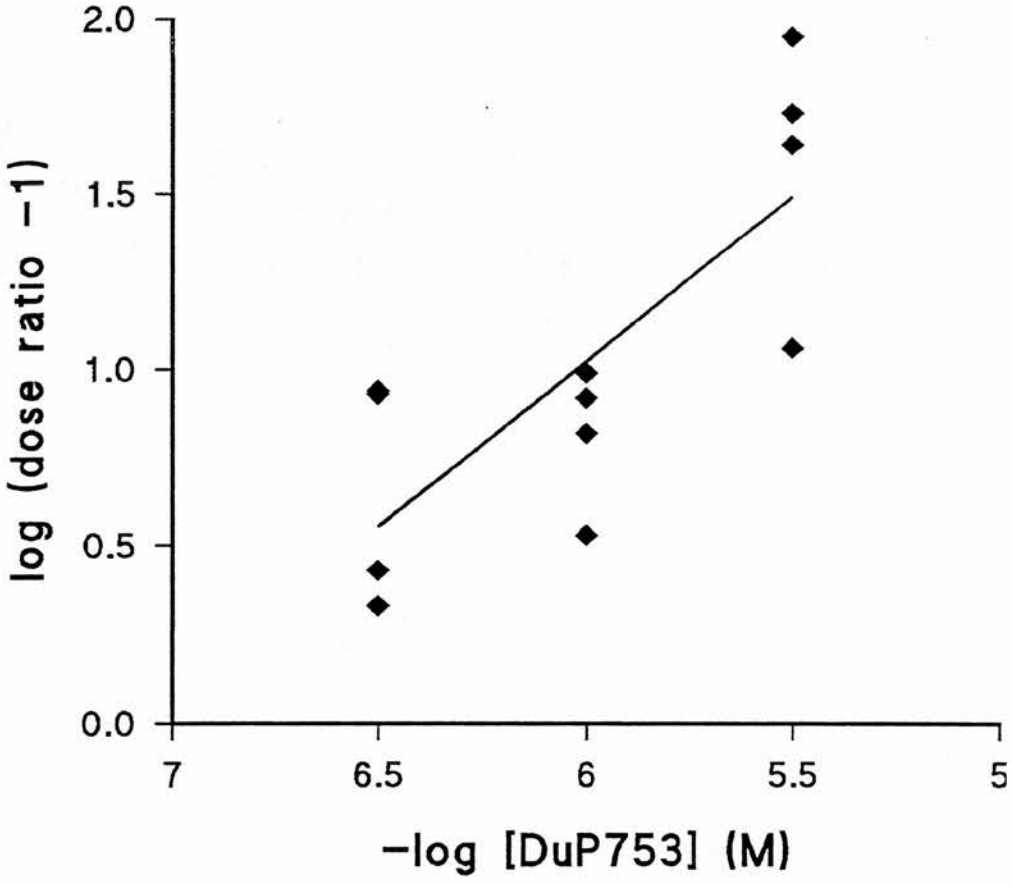


Figure 4.3

Schild regression analysis for DuP753 acting on AT_1 receptors in cultured bovine zg cells. Log (dr - 1) for the curves in Figure 4.2 and similar data from 3 further dose-response analyses is plotted against - log [DuP753]; the intercept on the x axis yields the pA_2 value for DuP753. The slope of the regression is 0.94 (95% C.I. 0.41-1.47) and the pA_2 (after constraint of the slope to 1) was calculated to be 7.02 (95% C.I. 6.82-7.22).

4.2.2 Schild regression analysis for zg cells

The Schild regression line for DuP753 in bovine zona glomerulosa cells is shown in Figure 4.3. The slope of the regression was 0.94 (95% C.I. 0.41-1.47). As this was not significantly different from unity, the slope was constrained to unity as the variation observed could be accounted for by chance (Mackay, 1978). The pA_2 obtained from this constrained plot was calculated to be 7.02 (95% C.I. 6.82-7.22) (Table 4.1).

4.2.3 Dose-response curves in vsm cells

Inhibition of AII-stimulated PI turnover in cultured rat mesenteric artery cells was studied in order to determine concentrations of DuP753 to use in Schild regression analysis. Figure 4.4 shows a representative experiment. Cells were plated into 24-well plates at 50,000 cells/well and allowed to grow to confluence. They were then labelled with ^{3}H-inositol as described in 2.2.2.6.1 for 72h prior to experiments carried out as in 2.2.2.6.2. After the addition of LiCl and unlabelled inositol to the cells, DuP753 was incubated with the cells for 5 minutes before addition of AII, in order to allow the antagonist to bind to the receptor. The cells were stimulated with AII for 20 minutes after which time the phosphatidylinositols were extracted from the cells as in 2.2.2.6.3. Cells were used between passages four and seven.

The IC_{50} for DuP753 was found to be 3.3×10^{-7}M, identical to that in the zg cells. Concentrations of DuP753 one log unit either side of the IC_{50} were tested for ability to shift dose-response curves to AII to the right. Unusually, only concentrations lower than the IC^{50} were effective, with higher concentrations producing no further shift. The concentrations of DuP753 used for the Schild regression analysis were therefore 3.3×10^{-8}, 1×10^{-7} and 3.3×10^{-7}M DuP753.

Figure 4.5 shows dose-response curves to AII in the absence and presence of DuP753. The curves again shift rightwards in a parallel fashion, with no significant depression in the maximal response.

4.2.4 Schild regression analysis for vsm cells

The Schild regression line for DuP753 in cultured rat mesenteric smooth muscle cells was then plotted (Figure 4.6) using combined data from three similar experiments. The slope of the line was 1.61 (95% C.I. 0.99-2.22). Although this was not significantly different from unity, unity was at the extreme end of the confidence interval. For this reason, individual log K_B values were calculated from the log (dr - 1) values obtained for each concentration of DuP753 using the Schild equation, assuming a slope of unity. Nine such values were obtained, three for each DuP753 concentration. The values were then analysed by ANOVA. The log K_B values obtained for 3.3×10^{-8}M DuP753 were significantly different ($p<0.05$) from those obtained for the other two concentrations. This suggested that a Schild regression of slope constrained to unity would be invalid (Mackay, 1978), and the pA_2 was therefore calculated from the regression line as plotted in Figure 4.6. The pA_2 was found to be 7.28 (95% C.I. 7.05 - 7.51) (Table 4.1).

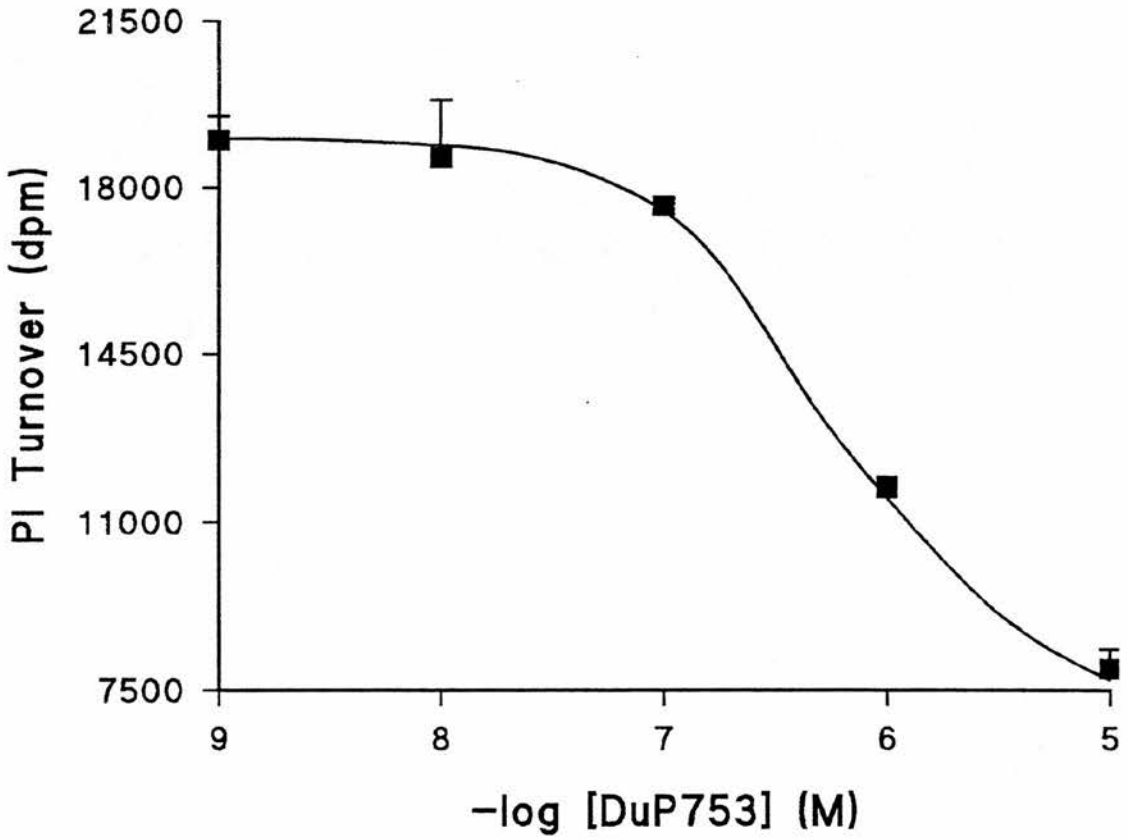


Figure 4.4

Determination of IC_{50} for DuP753 in cultured rat vsmc. Inhibition of AII-induced phosphatidylinositol turnover was measured (as total accumulated ^{3}H-phosphoinositols in the presence of a LiCl block) in ^{3}H-inositol labelled vsmc between passages 4 and 7. Cells were labelled for 72 hours before stimulation with 10nM AII in the presence of increasing concentrations of DuP753. The concentration of antagonist inhibiting maximal response by 50% was calculated to be 3.3×10^{-7} M. Representative data from one of two experiments.

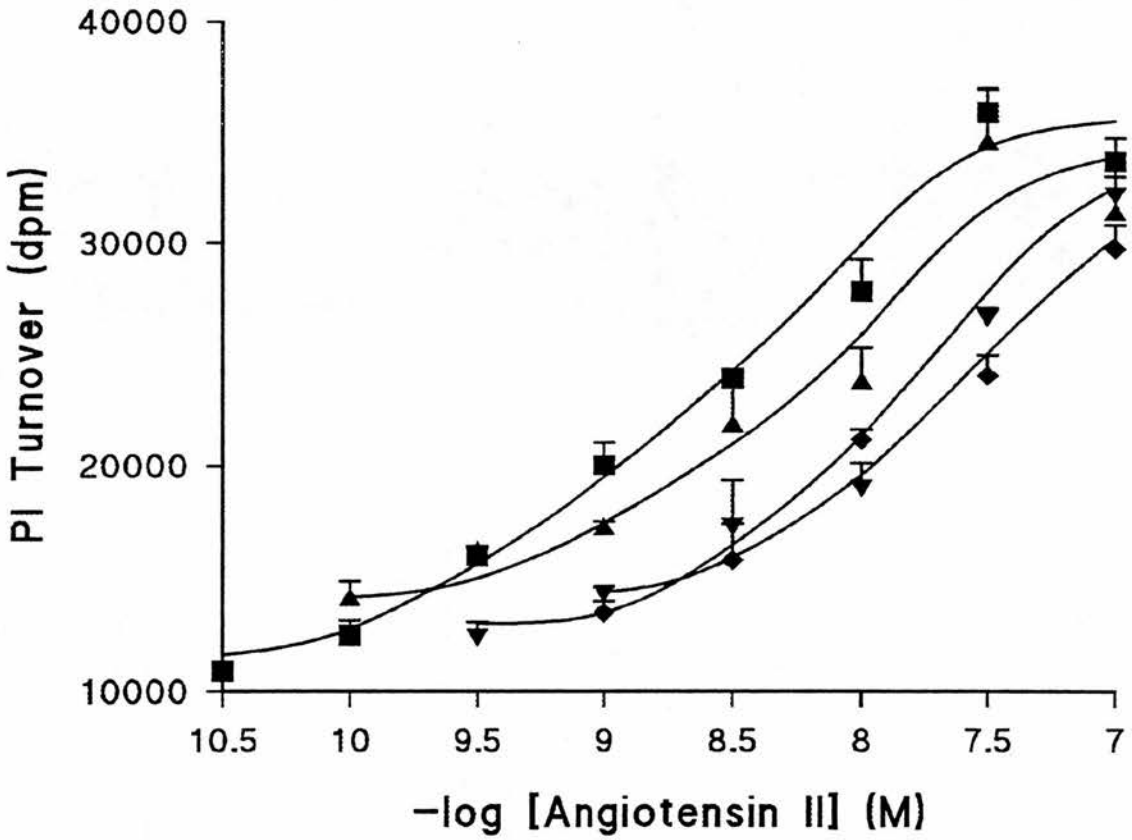


Figure 4.5

Dose-response curves to AII in cultured rat vsmc. Cells were labelled with ^{3}H-inositol for 72 hours and then stimulated for 20 minutes with varying doses of AII in the absence (■) or presence of 3.3 x 10^{-8} M DuP753 (▲), 1 x 10^{-7} M DuP753 (▼) or 3.3 x 10^{-7} M DuP753 (◆). PI Turnover measured as total accumulated ^{3}H-phosphoinositols in the presence of a LiCl block. Curves were shifted to the right in a parallel manner, with no significant depression in the maximal response. Representative data from one of three experiments.

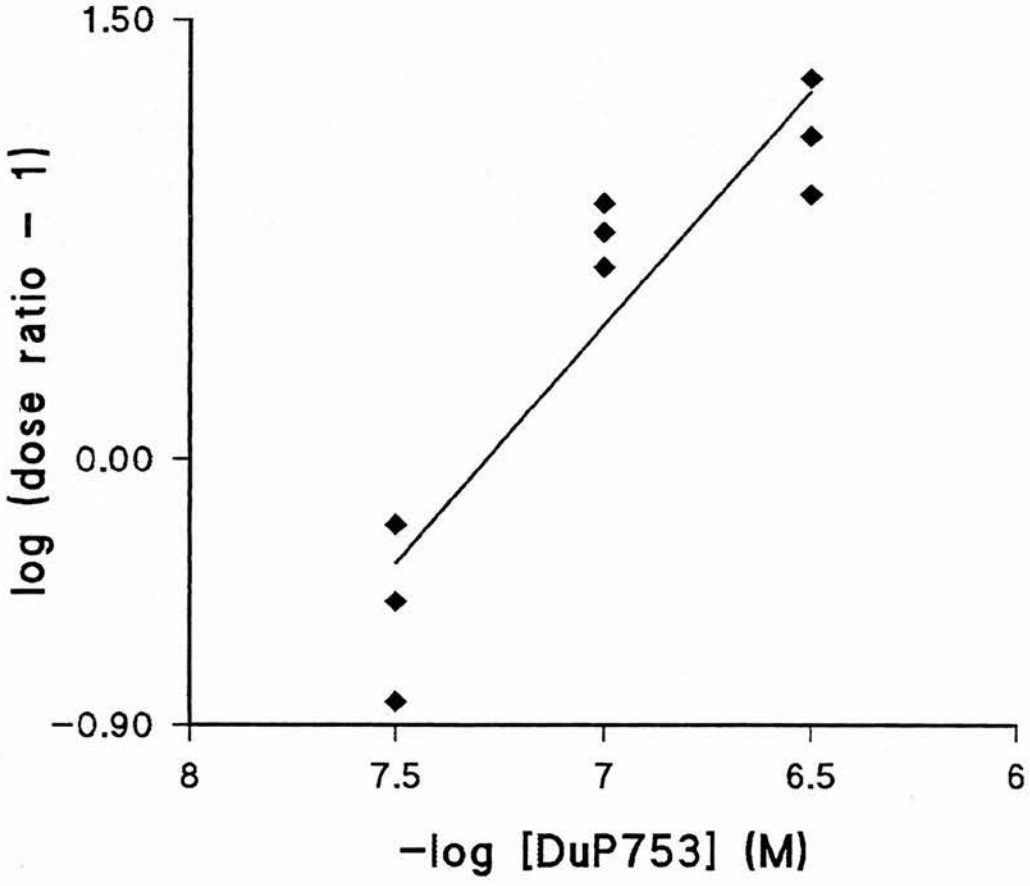


Figure 4.6

Schild regression line for DuP753 in cultured rat mesenteric artery smooth muscle cells. Axes are as for Figure 4.3; the slope of the regression is 1.60 (95% C.I. 0.99-2.41). The pA_2 for DuP753 was calculated from this regression line (from the intercept of the line at y = 0) and was found to be 7.28 (95% C.I. 7.05-7.51).

Tissue	pA_2 (experimental)	95% C.I. (experimental)	pA_2 (published)	95% C.I. (published)
Bovine zfr			7.02	6.89-7.15
Rabbit vsm			8.48	8.43-8.53
Bovine zg	7.02	6.82-7.22		
Rat vsmc	7.28	7.05-7.51		

Table 4.1

Comparison of experimental and published (Clyne *et al.* 1993; Chiu *et al.* 1990) values for pA_2 of DuP753 in adrenocortical cells and vascular smooth muscle. The 95% confidence interval is also shown. No significant difference was found between the experimental values measured in this thesis for bovine zg and rat vsmc, either between these cell types or when compared to the published values for bovine zfr cells. The pA_2 value published for vascular smooth muscle in the rabbit is, however, significantly different from the others ($p<0.001$ by ANOVA).

4.3 Discussion

Determination of pA_2 values for the AT_1 receptor antagonist DuP753 by Schild analysis in bovine adrenal zg and rat mesenteric artery vsm yielded values which were not significantly different from one another. This raises a number of points both about AII receptors and about their classification. An early indication that the receptors in the bovine adrenal zona glomerulosa and the rat mesenteric artery smooth muscle were not pharmacologically different was suggested by the identical IC_{50} values estimated for DuP753 in the two tissues. Pharmacologically distinct receptors would be expected to vary in this respect. There was, however, differing sensitivity in the two tissues, both in response to AII and in the concentration of DuP753 producing a rightwards shift. In the zona glomerulosa, concentrations lower than the IC_{50} for DuP753 produced no significant shift, whereas in the vascular smooth muscle, concentrations higher than the IC_{50} elicited no further shift. The EC_{50} for AII was also one log unit less sensitive in the rat mesenteric artery vsmc, being 3.3×10^{-8}M compared to 3.3×10^{-9}M in the bovine zg.

A possible explanation for this is the different responses used to quantitate the shifts. In the vsm cells, the second messenger response was used as an index for receptor activation. Stimulation of PI turnover is only part of the functional response to AII in vsmc and, for example, a sustained influx of Ca^{2+} may also be necessary to produce a full end-organ response. Therefore, in these experiments it is possible that the full magnitude of the end-organ response is not being reflected in the measurement made. This may explain why the dose ratios obtained for the vsmc are lower than for the zg cells, and would support the hypothesis that higher dose ratios are possible over a wider range of antagonist concentrations when the true end-organ response is used (as was the case when aldosterone secretion was measured in bovine zg cells). These different approaches may also account for the difference in EC_{50} to AII observed in the two tissues. Because the true

end-organ response is being measured for bovine zg cells, it is possible that a lower concentration of AII would be sufficient to elicit a half-maximal response than for the measurement of PI turnover in rat mesenteric artery vsmc. In this tissue, a higher concentration of AII would be required to elicit the half-maximal response, as the measurement of response precedes the full end-organ response of contraction.

There was a degree of scatter in the Schild regression plots for both tissues, which may reflect use of data from multiple cell preparations, which may have shown differing sensitivities to AII and DuP753, and also differing basal responses. The effect of any experimental error may also have been multiplied. The most obvious consequence of this was the large confidence intervals obtained for the slopes of the regression lines. For the zg Schild regression, the slope was almost identical to unity (0.94), indicating a true competitive antagonism of the DuP753 and that equilibrium had been reached between receptor, agonist and antagonist during the analysis. As the slope was not significantly different from unity, according to Mackay (1978), the variation may be attributable to chance. Thus, a Schild regression line of slope constrained to unity was plotted and the line extrapolated to the abscissa to obtain the pA_2. This was calculated to be 7.02 (Table 4.1).

This value was identical to that obtained for bovine adrenal zfr cells in culture (Clyne *et al.* 1993), providing evidence that the AT_1 receptors in the two zones are identical pharmacologically.

The slope for the vsmc Schild regression was 1.6. Though high, the value was not significantly different from unity, although unity was at the far extreme of the slope confidence interval. To determine whether the regression plot could validly be constrained to 1, individual log K_B values were calculated using the Schild equation and were found to vary significantly from 3.3×10^{-8}M to 1×10^{-7}M DuP753. On this basis, the Schild regression line could not be constrained and the plot shown in Figure 4.6 was used to calculate the pA_2, which was found to be 7.28 (Table 4.1). There are two possible explanations for the increased slope. One is that DuP753

was not acting competitively in this system. This is not likely since DuP753 has been shown to be a competitive antagonist in all other systems examined (Wong *et al.* 1990; Iwai *et al.* 1991). A second possibility is a failure to achieve equilibrium between the receptor, agonist and antagonist. The equilibrium is both time- and dose-dependent and therefore will be established more quickly for higher doses than lower ones. If true equilibrium has not been reached, the effect of low doses of antagonist will be underestimated and will therefore lead to a steepening of the Schild regression slope (Kenakin, 1987). It is also possible that, as DuP753 was added to the reaction before AII, AII might not have been able to equilibrate with the receptor to the same extent as the DuP753; this could lead to a failure of AII to displace the antagonist and thus a distortion of the results.

In the case of the zg, the duration of the experiment was 3 hours to allow aldosterone to accumulate to measurable levels. In the case of the vsmc, however, second messenger turnover was the measured variable. The labelled phosphoinositols accumulate linearly over a restricted time-course, dictating the selection of an early time-point - in this case, 20 minutes. It is possible that this time was insufficient to ensure that full temporal equilibrium was reached for DuP753 at the lowest dose.

Experimental pA_2 values obtained in this thesis for both bovine adrenal zg and rat vascular smooth muscle tissues were not significantly different. This implies that the AT_1 receptors in these tissues are the same. However, they were different from that published for vascular smooth muscle.

Therefore, the data in this thesis is at odds with the previously published pA_2 value for DuP753 acting on rabbit aortic smooth muscle (Chiu *et al.* 1990).

According to Arunlakshana and Schild (1959) and Mackay (1978), a pA_2 value should be constant for a particular receptor-antagonist coupling and variations should not be great between laboratories. In rats, which have been found to contain two AT_1 receptor subtypes, the subtypes have also

been shown to be pharmacologically indistinguishable from each other and the human AT_1 receptor (Balmforth *et al.* 1994) by a range of antagonists in transfected COS cells. DuP753 is selective for the AT_1 receptor, which is the only receptor type known to mediate the measured physiological responses to AII. Therefore, the differences in pA_2 cannot be explained by a difference in receptor population (e.g. the presence of AT_2 receptors). It is therefore necessary to consider the methods used for analysis of the AT_1 receptor antagonism by DuP753 in the two instances. The previously reported pA_2 value for rabbit aortic vsm was obtained using intact vessel segments in organ bath studies, whereas the value reported here for rat mesenteric artery was obtained from cultured cells. These two methods are quite different and possibly not directly comparable; it is possible that this variation may have contributed to the difference in pA_2 values observed. For instance, the culture conditions the cells were exposed to may in some way have changed the affinity of the receptor for the antagonist. Such a change could affect a measured pA_2. Mackay (1978) also mentions the importance of using similar systems when comparing pA_2 values for receptor classification purposes.

It would seem, therefore, that although Schild analysis is a useful and powerful tool in the classification of receptors by using selective antagonists, variations in experimental procedures may affect the resulting values. It is therefore important that the pA_2 values obtained are truly comparable.

In this chapter, the AT_1 receptors present in cultured bovine adrenal zona glomerulosa cells and cultured rat mesenteric artery smooth muscle cells have been pharmacologically characterised by Schild analysis. This analysis yielded pA_2 values for the antagonist DuP753 acting at the AT_1 receptor. The pA_2 values obtained were found to be not significantly different, indicating that the receptors are therefore pharmacologically indistinguishable. The pA_2 value for DuP753 acting at the AT_1 receptor in the zg was also compared to the corresponding pA_2 in the zfr. Both pA_2 values were again found to be not significantly different. These findings challenge earlier evidence (Chiu *et al.* 1990; Clyne *et al.* 1993) that a difference exists

between pA_2 values in the adrenal cortex and vascular smooth muscle, and highlights a possible pitfall in comparisons of pA_2 values.

Chapter 5 : Isolation and generation of a clone from the bovine adrenocortical AT_1 receptor coding region by PCR amplification

5.1 Introduction

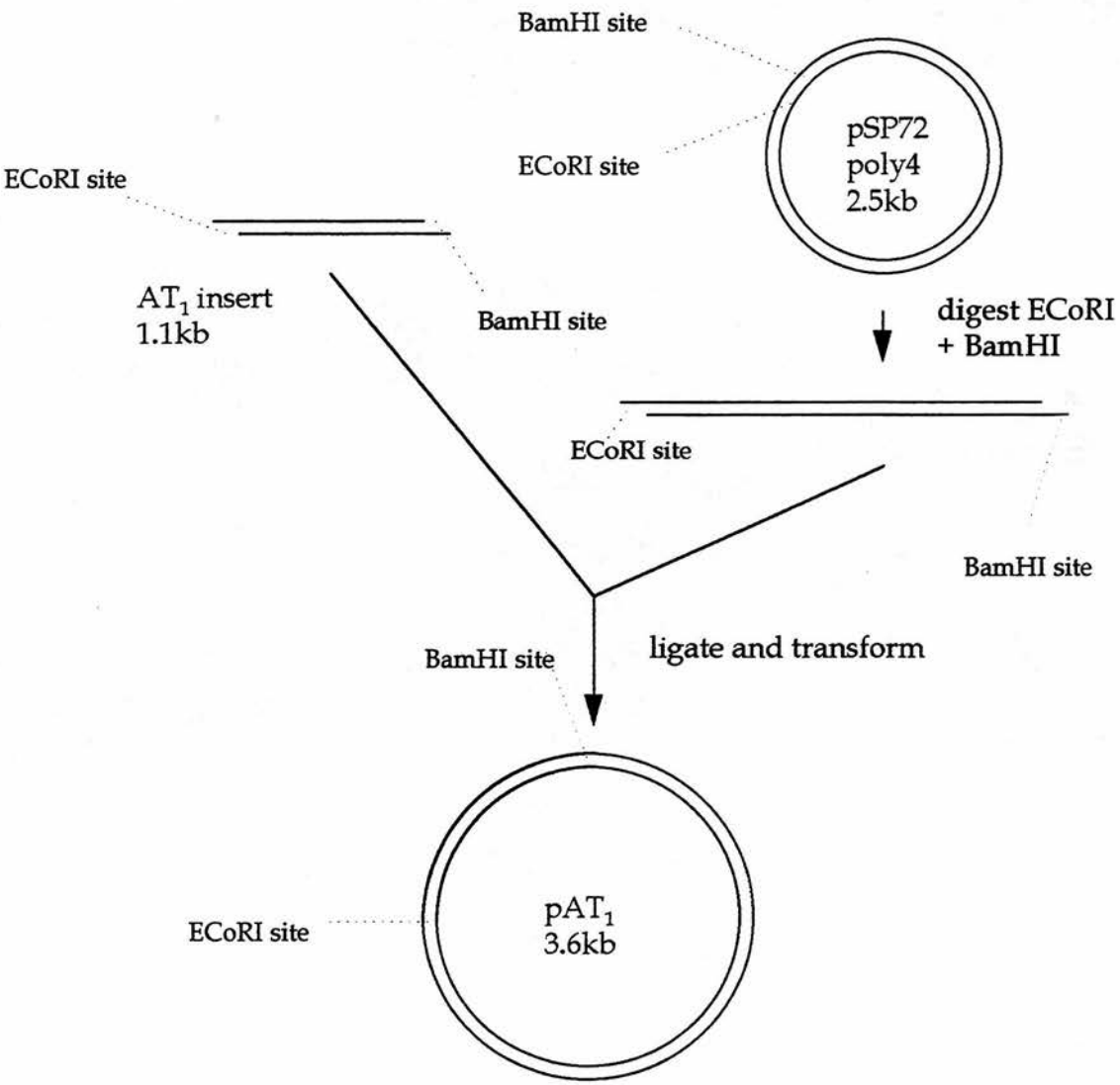
One of the aims of this thesis was to study the regulation of the AT_1 receptor. We wished to do this by analysing AT_1 receptor mRNA levels using Northern blotting, the simplest method of examining steady-state mRNA levels. To perform such an analysis, a probe recognising (i.e. complementary in sequence) the AT_1 receptor mRNA must be generated. The aim of this chapter, therefore, was to PCR-amplify the coding region of the bovine adrenocortical AT_1 receptor (Sasaki *et al.* 1991), and from this amplified sequence to generate a clone of the AT_1 receptor.

We chose this approach because of its simplicity and the knowledge that the bovine AT_1 receptor contained no introns in its coding sequence (Dr. T. Inagami, personal communication). PCR primers were designed against the coding region of the published sequence for the bovine adrenal AT_1 receptor, and used to amplify a 1.1kb fragment from bovine genomic DNA. This fragment was then inserted into a plasmid vector and subjected to restriction mapping to confirm its identity with the AT_1 coding region sequence. This cloning strategy can be seen in Figure 5.1. Further confirmation having been provided by full sequence analysis of the clone and comparison with the published sequence, the clone was then successfully used to generate a probe for use in Northern blot analysis of the bovine adrenal AT_1 receptor.

Figure 5.1

Cloning strategy for the bovine adrenal AT_1 receptor coding region PCR fragment. The amplified PCR fragment ① is represented following concatemerisation and restriction to produce EcoRI/BamHI compatible 'sticky ends', i.e. ready for insertion into the pSP72poly4 vector. Closed circular pSP72poly4 ② is linearised by restriction digest to yield EcoRI and BamHI compatible ends ③. The AT_1 receptor coding region PCR fragment is then inserted into the cloning vector ④, followed by transformation into *E.coli DH5*. AT_1 receptor transformants are then selected by growth on ampicillin-containing agar plates. The position of restriction sites used in the cloning are shown.

AT_1 CLONING


BamHI site
ECoRI site
pSP72
poly4
2.5kb
ECoRI site
AT_1 insert
1.1kb
BamHI site
digest ECoRI
+ BamHI
ECoRI site
BamHI site
BamHI site
ligate and transform
ECoRI site
pAT_1
3.6kb

5.2 Results

5.2.1 PCR of the AT_1 receptor coding region sequence

In order to amplify the coding region of the AT_1 receptor, primers were designed against the published sequence ((Sasaki *et al.* 1991), Figure 5.2). Since it had previously been established (Dr. T. Inagami, personal communication) that the AT_1 receptor contained no introns in the coding region, primers were designed to encompass the whole of this region, with 5 (5′) and 13 (3′) flanking bases on each side. The primers were 29 (5′) and 32 (3′) bases in length, with 23 bases homologous to the AT_1 sequence. An EcoRI site was included in the 5′ primer, and a BamHI site in the 3′ primer to permit easier cloning by generation of non-compatible 'sticky ends'. Both primers included a CG clamp (three or more C or G residues at the 5′ end of the primers) to anchor them more firmly to the DNA template by hydrogen bonding. The locations of the primers and the additional bases incorporating the restriction sites are shown in Figure 5.2.

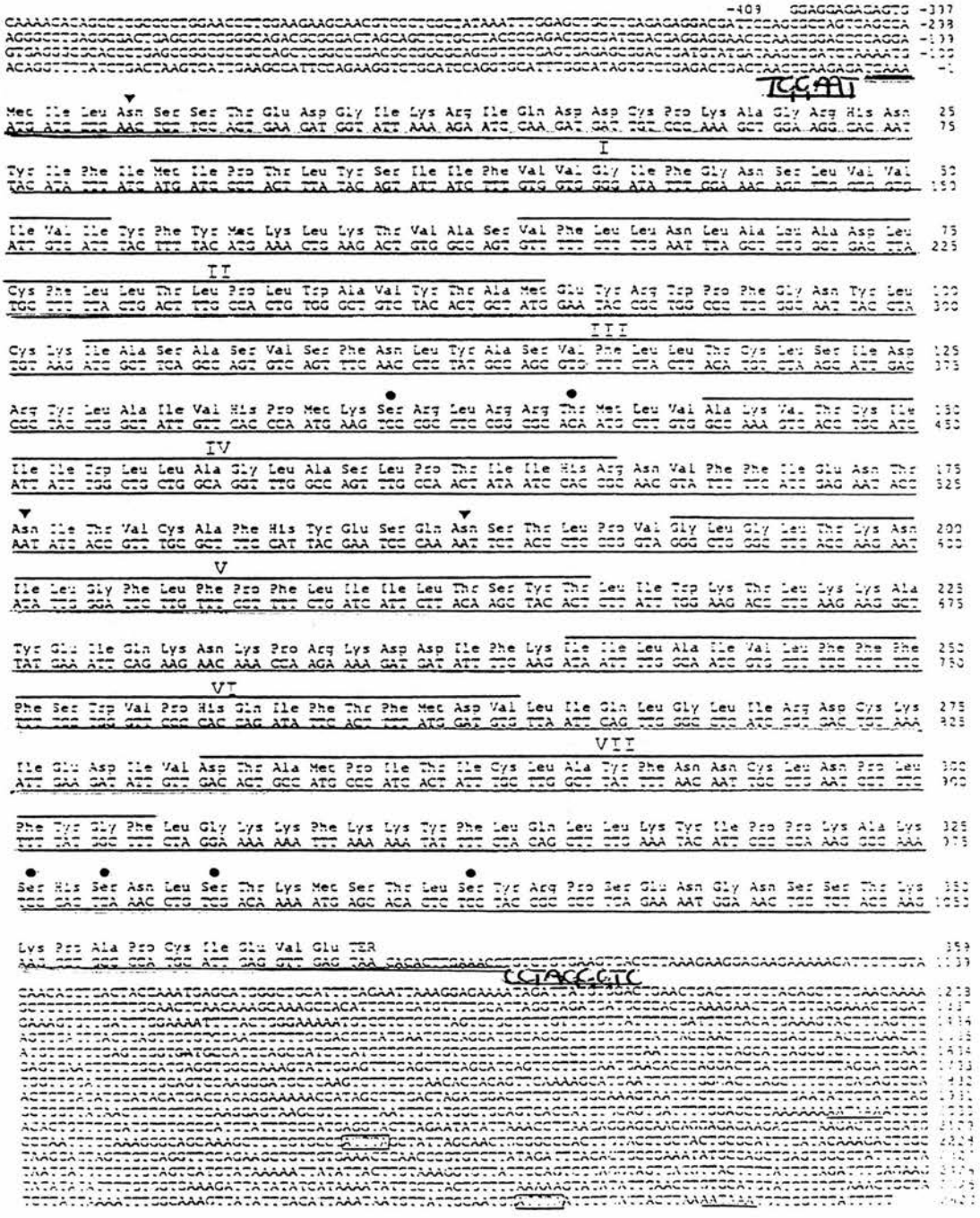
After optimisation of the PCR (2.2.3.5), microgram quantities of the 1114bp AT_1 fragment were successfully amplified using the following conditions : annealing temperature 65°C; initial DNA (purified from bovine spleen) 0.02μg; Taq DNA polymerase; 32 cycles. The resultant DNA was split into two pools which were purified by one of two methods (2.2.3.5).

5.2.2 Purification of the AT_1 PCR fragment for cloning

After purification, the two pools of AT_1 PCR fragment were concatemerised (2.2.3.6) and restricted (2.2.3.7) to generate DNA with 'sticky ends' for ligation into the cloning vector. Aliquots of the two pools of DNA were taken following concatemerisation and restriction and run on

Figure 5.2

Published sequence of the bovine adrenal AT_1 receptor (taken from Sasaki *et al.* 1991). The coding region and its predicted amino acid sequence are shown with the 5′ and 3′ flanking sequences of the cDNA clone. Underlined in green are the sequences used for the PCR primers; added, also in green, are the nucleotides comprising the added restriction sites (5′ EcoRI; 3′ BamHI), which are additionally highlighted. Underlined in red is the sequence which was amplified, an amplimer of 1114bp.



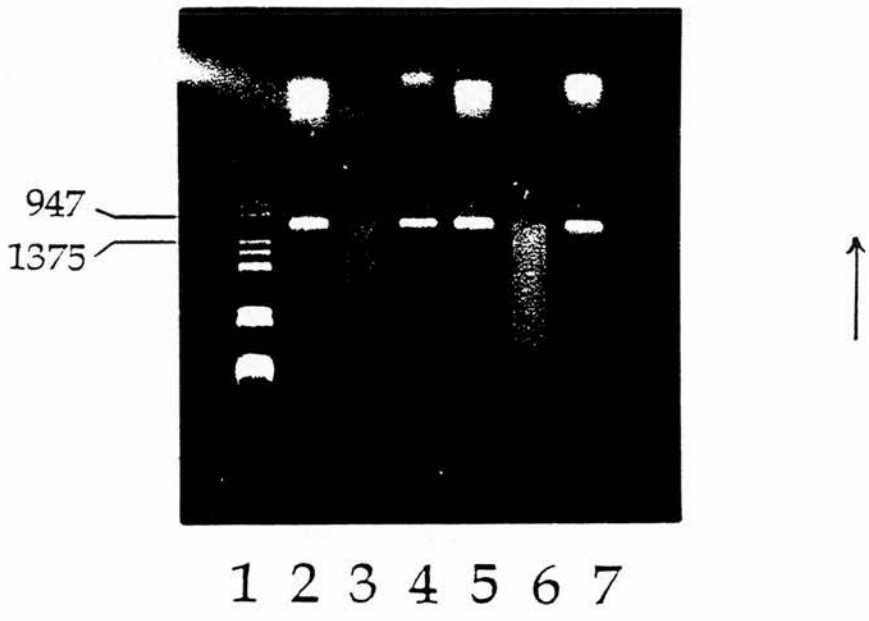


Figure 5.3

Purification of the amplified AT_1 fragment for cloning. The ethidium bromide-stained 0.7% (w/v) agarose gel has been run bottom to top as indicated by the arrow. Lane 1 contains λ (HinDIII/EcoRI digest : 2.2.3.4.2) DNA size markers; the appropriate sizes are indicated. Lanes 2-4 and 5-7 are from AT_1 pools ① and ② respectively. Lanes 2 and 5 : PCR fragment, size ~ 1.1kb; lanes 3 and 6 : AT_1 fragment after KKL procedure to generate concatemers; lanes 4 and 7 : AT_1 fragment after restriction digest with EcoRI and BamHI to regenerate monomeric AT_1 fragments ~1.1kb in length.

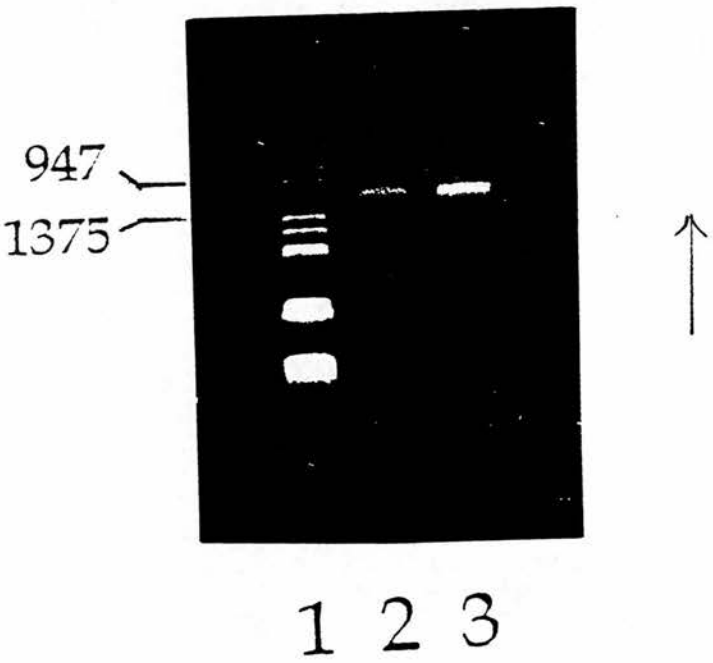


Figure 5.4

Ethidium bromide-stained 0.7% (w/v) agarose gel showing the two pools of AT_1 PCR fragment after gel purification and subsequent extraction. Lane 1 shows λ (HinDIII/EcoRI digest) DNA markers; the sizes of the relevant bands are shown alongside. Lanes 2 and 3 are the pool ① and ② AT_1 fragments, ~1.1kb in length, in both cases.

a 0.7% (w/v) agarose gel (Figure 5.3).

The figure is in two parts; the left three bands representing pool ① and the right pool ②. The first band in each set (lanes 2 & 5) is the AT_1 fragment, of the correct size (~1.1kb), after PCR. Clean, sharp bands are present in each, indicating successful amplification of the desired product and the absence of non-specific background. Some unamplified primers can be seen at the top of each lane. The next set of bands (lanes 3 & 6) is the AT_1 fragments after KKL concatemerisation. The concatemers are represented by the band smearing down the gel, which indicates concatemers of increasing length. The longer trailing band in pool ② suggests that the DNA fragments in this pool have been more extensively concatemerised.

The third pair of bands (lanes 3 & 7) shows the 1.1kb AT_1 fragment regenerated after restriction digest of the concatemers with EcoRI and BamHI, as in 2.2.3.7. The bands are again sharp and clear, denoting full breakdown of the concatemers. At this stage, before gel purification of the majority of the AT_1 receptor PCR fragments, small aliquots of the DNA were taken for use in ligations (AT_1 ① and ② 'pre').

Figure 5.4 shows the appearance of the AT_1 fragments following electrophoresis to remove excess PCR primers and recovery from an agarose gel (2.2.3.8). As before, the fragments from the two pools are shown. The bands are sharp and of the correct size. The band from pool ② is of greater intensity than that from pool ①, indicating a greater degree of recovery in the second pool. The primers have also been completely removed by the gel purification. Aliquots of this gel-purified DNA were then used in ligations (AT_1 ① and ② 'post').

5.2.3 Choice and preparation of cloning vector

The vector chosen for the cloning of the AT_1 receptor sequence was pSP72poly4, a modified version of pSP72 (Promega) containing an altered polylinker (S. Morley, unpublished) (Figure 5.5). It was chosen because of the availability of

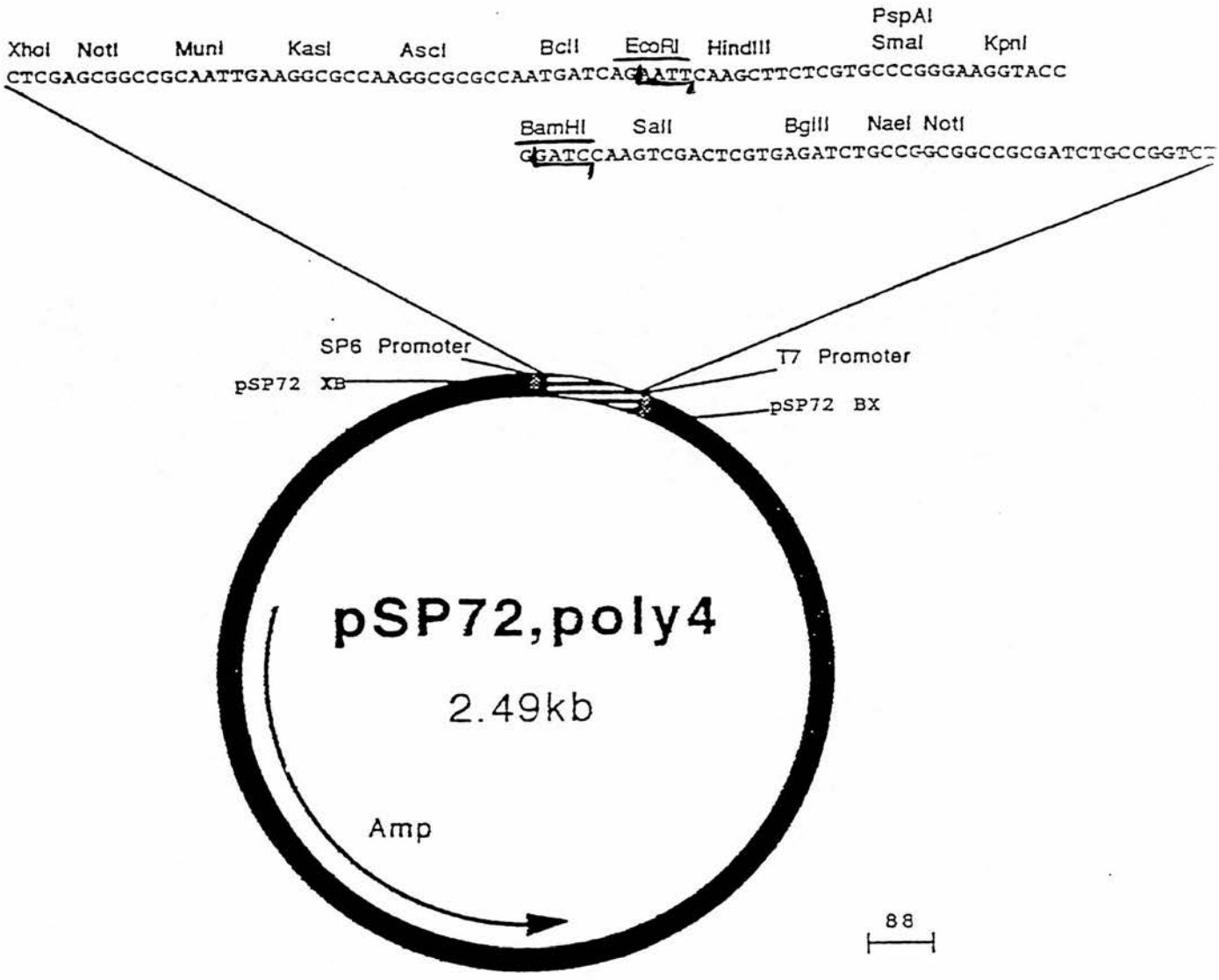


Figure 5.5

pSP72poly4, the plasmid vector used for cloning the AT_1 receptor sequence. This vector, derived from the Promega vector pSP72 contains a modified polylinker (S.D. Morley, personal communication) : the nucleotide sequence shown containing multiple restriction sites. Among these sites are highlighted EcoRI and BamHI, which are compatible with sites designed onto the AT_1 PCR fragment via the PCR primers. Red lines show the points of cutting to generate 'sticky ends' for insertion of the AT_1 fragment. The vector also contains a gene for ampicillin resistance, which was used to select for bacteria transformed with the assembled cloning vector. Universal sequencing primers are also indicated (pSP72XB and pSP72BX).

EcoRI/BamHI insertion sites, compatible with the sites added to the AT_1 sequence by the PCR primers, and the ampicillin selection facility, as well as its small size and high copy number on transformation into *DH5 E.coli* cells. Proven sequencing primers were also available flanking the insertion site. The vector was prepared for use by digesting with EcoRI and BamHI, followed by gel purification and extraction as detailed in 2.2.3.8. The use of two restriction enzyme sites for cloning minimised the risk of vector re-circularisation during ligation, as well as allowing unambiguous direction of insertion of the AT_1 receptor sequence.

5.2.4 Cloning strategy and procedure

The cloning strategy employed is shown in Figure 5.1. AT_1 insert (from the two pools at different stages) and pSP72poly4 vector were ligated in varying proportions (5:1 and 20:1 insert:vector on a molar basis, using 10ng vector), before transformation into the host bacteria (2.2.3.9, 2.2.3.11). Transformants were selected by growth of colonies on L-agar plates containing ampicillin. Table 2.1 (see Methods) shows the transformation efficiencies for the different ligation reactions. Ligations from AT_1 ① 'post' 20:1, AT_1 ② 'post' 5:1 and 20:1 yielded higher transformation efficiencies than the other groups. 5 Sample colonies were taken from each plate to test for incorporation of successfully ligated insert and vector.

5.2.5 Confirmation of positive colonies

Plasmid DNA was prepared from 50 sample colonies (2.2.3.12) and digested with EcoRI and BamHI to detect the presence of an incorporated insert (data not shown). Of these, 12 clones shown to contain a 1.1kb insert were re-analysed on a 0.7% (w/v) agarose gel, shown in Figure 5.6, and retained for further analysis. Both the plasmid band (2.5kb) and insert band (1.1kb) are clearly visible. Positive and negative controls are also shown. The

frequency of successful ligation is shown in Table 2.1. It was highest in AT_1 ② 'post' 5 and 20:1, where all colonies picked were positive for the AT_1 insert, showing the increased efficiency of this purification method. Of the colonies sampled, twelve were found to be positive for a 1.1kb insert.

5.2.6 Restriction mapping of positive AT_1 clones

Analysis of the published AT_1 receptor sequence (Sasaki *et al.* 1991) indicated the presence of putative restriction sites for BSpMI, DraI, MunI, SalI and SSpI within the coding sequence at positions 451, 926, 881, 989 and 933 respectively. Since all of these enzymes also restrict pSP72poly4 at known sites, digestion of a pSP72poly4 recombinant plasmid containing the EcoRI/BamHI AT_1 receptor PCR fragment should yield diagnostic restriction fragments of predictable sizes. DNA from each of the twelve clones in Figure 5.6 were therefore digested with these enzymes and the resultant fragments resolved on a 0.7% (w/v) (Figure 5.7; a 2% (w/v) gel was used for resolution of the 100bp SalI fragment).

Ten of the twelve clones analysed displayed the predicted pattern of restriction sites and were thus tentatively identified as AT_1 receptor clones (Table 5.1). The two other clones (pAT_1.43 and 50) were unusual. MunI digestion of pAT_1.43 yielded only one fragment, indicating the absence of this site in the insert, while digestion with BSpMI, DraI and SSpI yielded the predicted number of fragment. These, however, differed slightly in size from what was expected, as did fragments resulting from digestion of pAT_1.50 with BSpMI, DraI, MunI and SSpI. The SalI site, however, was present in the expected position in both clones.

Four of the clones were selected for further analysis : pAT_1.2C, and 44 were selected as representative of the putative AT_1 receptor PCR clones, while pAT_1 43 and 50 were analysed to determine why they yielded anomalous restriction maps.

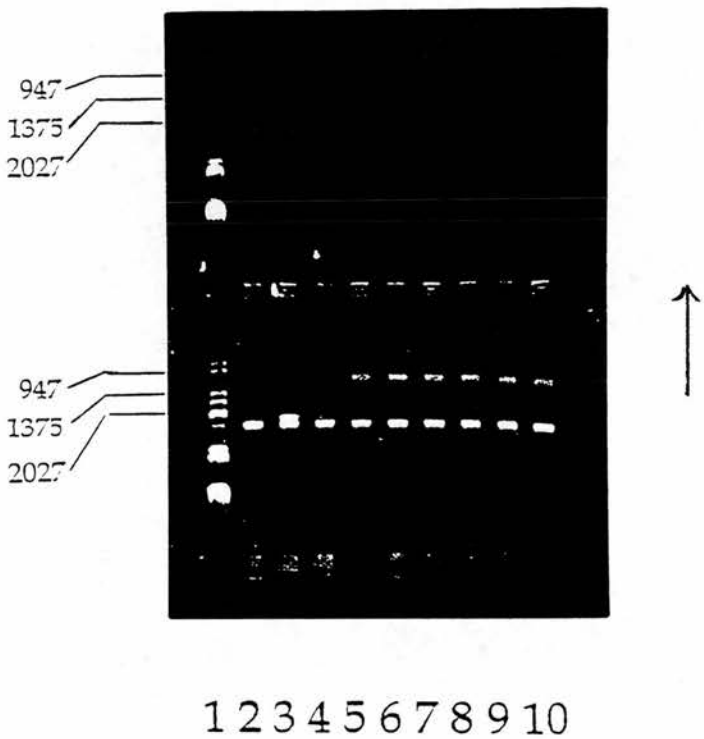


Figure 5.6

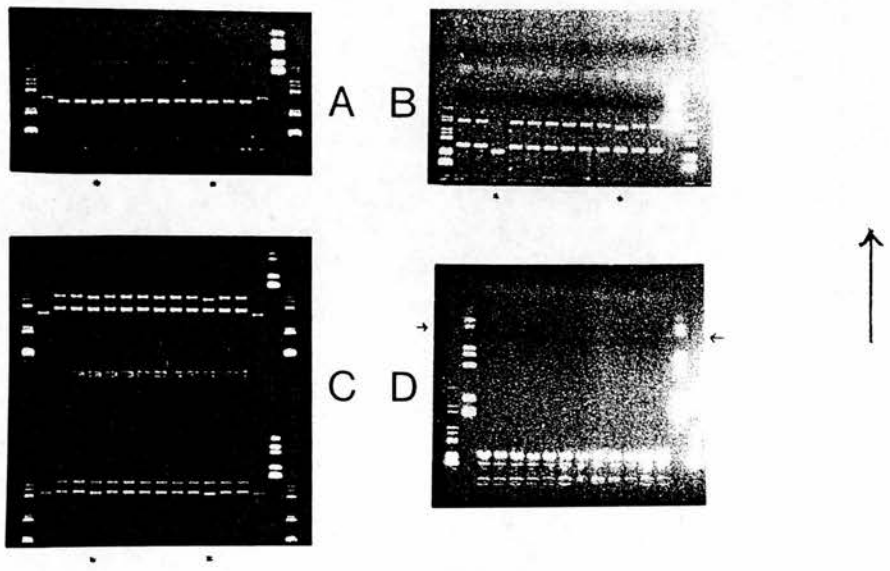
Confirmation that colonies after transformation contained AT_1 inserts. Plasmid DNA was prepared from sample colonies picked from all transformation plates (2.2.3.12) and digested with EcoRI and BamHI to liberate any inserts from the vector. Those found to be positive (i.e. to contain the 1.1kb insert) are shown on this ethidium bromide-stained 0.7% (w/v) agarose gel, with negative and positive controls. The top and bottom portions of the gel contain the same markers and controls, with different test clones. Top : lane 1 : λ (HinDIII/EcoRI digest) DNA size markers, lane 2 ; negative (vector only), lane 3 : positive (vector + 2.7kb control insert), lane 4 : negative (vector only), lanes 5-10 : positives 2C, 26, 43, 44, 45, 46. Bottom : lanes 1-4 as top, lanes 5-10 : positives 47, 48, 49, 50, 51, 52. AT_1 receptor insert bands are the expected (~1.1kb) size.

Figure 5.7

Restriction mapping of the twelve pAT_1 clones. After confirmation of the expected insert size (1.1kb) by EcoRI/BamHI digestion, plasmid DNA was digested with five restriction enzymes diagnostic for the AT_1 receptor coding sequence : BSpMI (A); MunI (B); SSpI and DraI (C upper and lower); and SalI (D). All of these enzymes also had restriction sites within pSP72poly4. Consequently, if the 1.1kb insert was indeed an AT1 receptor coding region, digestion with these enzymes would liberate a diagnostic pattern of predictable fragment sizes as follows : BSpMI : 2.8kb and 735bp; MunI : 2.7kb and 914bp; SSpI : 2.1kb and 1.4kb; DraI : 1.7kb, 1.2kb and 700bp; SalI : 3.5kb and 100bp. Arrows indicate the position of the smaller SalI fragment on panel D. Two of the clones (pAT_1.43 and 50) consistently showed abnormalities in the restriction sites for the first four enzymes; these are indicated with asterisks. Gels A and C were run as follows : λ (HinDIII/EcoRI digest) DNA size markers in the left-and righthandmost lanes, Bluescript $BSSK^+$ DNA markers (low size) in the right-hand penultimate lane. The putative pAT_1 clones were run in the order 2C, 26, 43-52, flanked on either side by pSP72poly4 alone as a control. Panels B and D are identical except that no negative controls were run, and D has an extra lane of Bluescript markers in the left-hand penultimate lane.

Table 5.1

Restriction mapping of the twelve pAT_1 clones. This table summarises the information shown in the above figure. Restriction sites : Y=present; N=absent, ?=abnormal.



(pAT$_1$.n)	SalI	MunI	BSpMI	DraI	SSpI	pAT$_1$.n ?
2C	Y	Y	Y	Y	Y	Y
26	Y	Y	Y	Y	Y	Y
43 *	Y	X	?	?	?	N
44	Y	Y	Y	Y	Y	Y
45	Y	Y	Y	Y	Y	Y
46	Y	Y	Y	Y	Y	Y
47	Y	Y	Y	Y	Y	Y
48	Y	Y	Y	Y	Y	Y
49	Y	Y	Y	Y	Y	Y
50 *	Y	?	?	?	?	N
51	Y	Y	Y	Y	Y	Y
52	Y	Y	Y	Y	Y	Y

5.2.7 Sequencing of the pAT_1 clones

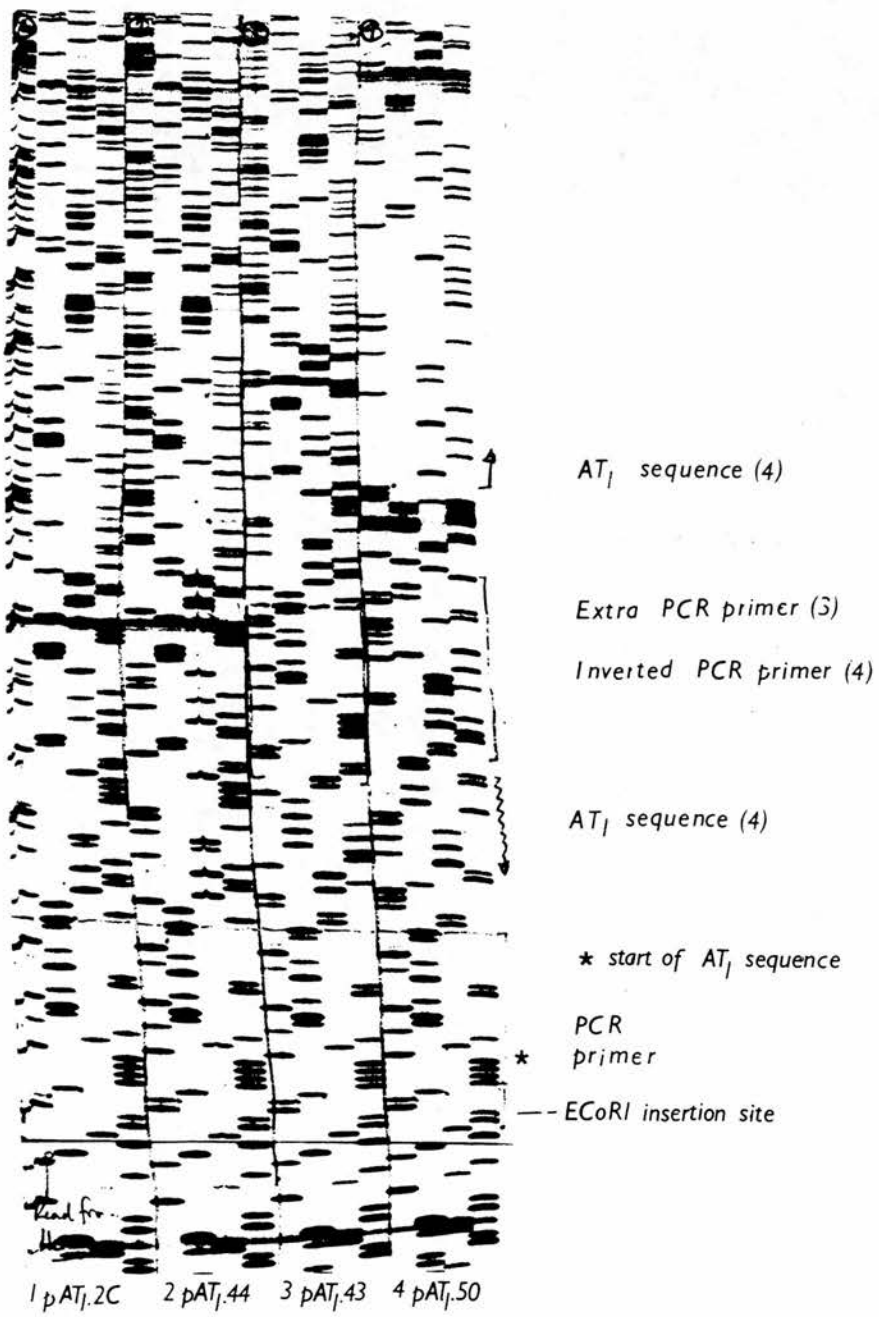
DNA from large-scale preparations of pAT_1.2C, 44, 43 and 50 was purified by CsCl gradient centrifugation (2.2.3.13), denatured and sequenced by the dideoxynucleotide method (2.2.3.14). A typical sequencing gel showing 5′ sequence from each of the clones is shown in Figure 5.8. This gel employed a sequencing primer designed to hybridise to the cloning vector (pSP72XB); subsequently the rest of the length of the AT_1 insert was sequenced by the technique of 'primer walking', where new sequencing primers were designed against the far end of the sequence obtained from the previous primer. The sequences of the primers used are shown in 2.2.3.14, in order of use : the pSP72XB and BX are shown first for each direction, followed by the PCR primers and then additional primers.

Comparison of the sequences for pAT_1.2C, 44, 43 and 50 shown in Figure 5.8, with each other and with the published sequence for the bovine adrenal AT_1 receptor immediately suggested an explanation for the anomalous restriction maps for clones pAT_1.43 and 50. The majority of sequence for both clones was clearly derived from the bovine AT_1 receptor, but pAT_1.43 contained two copies of the 5′ EcoRI PCR primer separated by a small amount of unidentifiable sequence. Meanwhile, pAT_1.50 contained a small amount of inverted AT_1 sequence immediately after the 5′ EcoRI PCR primer, followed by an inverted copy of the 5′ EcoRI PCR primer and authentic AT_1 sequence. Approximately 50 and 80 extra bases were incorporated into pAT_1.43 and 50 respectively; these insertions would have produced the observed anomalies in the restriction maps. Since these clones obviously represented PCR amplification and cloning artefacts, they were not subjected to any further analysis.

Analysis of the sequences for pAT_1.2C and 44 (Figure 5.8) from the pSP72XB sequencing primer indicated that they represented authentic AT_1 receptor clones. Accordingly, the inserts in these clones were sequenced along their entire length on both strands, with the exception of two CG-rich regions where the sequence was highly compressed. These were

Figure 5.8

Sequencing gel from the analysis of pAT_1 clones; from left to right, pAT_1.2C, 44, 43 and 50. The base order, left - right, is TCGA. The primer used is the 5′ universal primer on pSP72poly4 (pSP72BX), yielding sequence encompassing the 5′ EcoRI cloning insertion site and the 5′ start of the AT_1 sequence. The start of the insertion site is clearly marked, as is the start of the AT_1 coding sequence. Clones pAT_1.2C and 44 contain no obvious differences; however, clone pAT_1.43 has an extra PCR primer incorporated into the sequence, as marked. pAT_1.50 contains an inverted PCR primer, some inverted AT_1 coding sequence, and then a correctly inserted PCR primer followed by the AT_1 coding sequence, further into the sequence. These are indicated on the figure.


AT$_1$ sequence (4)
Extra PCR primer (3)
Inverted PCR primer (4)
AT$_1$ sequence (4)
* start of AT$_1$ sequence
PCR primer
ECoRI insertion site
1 pAT$_1$.2C
2 pAT$_1$.44
3 pAT$_1$.43
4 pAT$_1$.50

Position (in published sequence)	Published	$pAT_1.2C$	$pAT_1.44$
481	C	T	
568	T		C
725	G		A
802	T		C
818 -	CGCCTCCGG		SKCCKR_ _ *
820 -	CCTCCGG	_ _TCCKS *	
913	C	T	T
990 -	GGCTGG	KYGTGK *	
991 -	GCTGGG		CTGGAG *
1015	G		A
1475	A	G	G

Table 5.2

Sequence discrepancies from the published sequence for the bovine adrenal AT_1 receptor (Sasaki *et al.* 1991). Base uncertainty codes are as follows : Y : C/T; S : G/C; K : G/T; R :A/G. Discrepancies marked with an asterisk indicate those from areas sequenced on one strand only due to unresolveable C-G compressions. (Further sequencing of these areas to yield uncompressed double-stranded sequence is anticipated).

unresolveable using dITP as a dGTP analogue and therefore these sequences were obtained for one strand only. Table 5.2 summarises the sequence discrepancies in the clones pAT_1.2C and 44 compared to the published sequence. The overall homology was 98.4% for clone pAT_1.44, and 98.9% for clone pAT_1.2C at the present state of analysis.

The sequence analysis indicated that the pAT_1 clones generated were in fact AT_1 receptors, and not of the AT_2 subtype. It also showed that the errors in the clones with abnormal restriction maps were due to misincorporation of PCR primers at the 5′ end. The degree of homology compared with the published sequence indicates that the products are, with the exception of the unresolved areas and single base misincorporations, true copies of the bovine adrenal AT_1 receptor.

5.2.8 Use of the AT_1 fragment to detect AT_1 mRNA

As described in section 2.2.3.15 and 16, the AT_1 coding region DNA generated by the cloning procedure was used as a probe in Northern blots to detect AT_1 receptor mRNA in cultured bovine adrenocortical cells. An example of an autoradiograph can be seen in Figure 2.4, where different probe preparation methods were compared for their sensitivity. The transcript size observed is the same as that published (Sasaki *et al.* 1991) : 3.3kb. The copy controls can also be clearly seen, indicating that in a 25μg sample of adrenal cell total RNA, as little as 5pg AT_1 receptor mRNA can be detected.

5.3 Discussion

We wished to generate a probe for the bovine adrenal AT_1 receptor to use in Northern blot analysis of AT_1 receptor mRNA steady-state levels. The sequence of the receptor had previously been published (Sasaki *et al.* 1991); the AT_1 receptor had also been shown to belong to a seven-transmembrane region family of G-protein coupled receptors which contain no introns in the coding regions of their genes (Dr. T. Inagami, personal communication). We therefore decided to clone the entire receptor coding sequence by PCR amplification. The amplification could be performed using bovine genomic DNA as the coding region sequence contained no introns.

Primers were therefore designed to a high degree of specificity against the AT_1 receptor coding sequence, encompassing the whole length of the coding region, with a little 5′ and 3′ flanking sequence. A CG clamp was designed onto the primer ends to facilitate efficient annealing; the primers were also designed to contain 5′ EcoRI and 3′ BamHI restriction sites to facilitate ligation into the cloning vector. On the first PCR amplification, therefore, the primers would not exactly fit with the genomic sequence; however, the noncomplementary 'tail' (see Figure 5.2) was short in comparison with the rest of the primer, in order to minimise any problems this might have caused.

After optimisation of the PCR conditions as in 2.2.3.5, a fragment of the expected size (1114bp = ~1.1kb) was successfully amplified, as shown in Figure 5.3. Two purification methods for the PCR fragment were then used to maximise the chances for successful ligation into the cloning vector. The second of these methods yielded longer concatemers, as shown in Figure 5.3, and DNA purified in this way also yielded more clones containing 1.1kb inserts (Table 2.1), leading to the conclusion that this purification method was more effective than that used for pool ①, possibly due to improved removal of enzymatic inhibitors.

Twelve colonies out of a total of 50 transformants tested were found to contain 1.1kb inserts (Figure 5.6). The identity of those inserts was confirmed by digestion of plasmid DNA isolated from these twelve colonies with five restriction enzymes known to have cutting sites within the AT_1 receptor sequence. Ten of these clones displayed restriction patterns consistent with identification as an AT_1 clone, while the other two were anomalous (Figure 5.7; Table 5.1).

These anomalies could have indicated one of two things : that several errors had occurred during the amplification process, or alternatively that a second AT_1 receptor subtype had been cloned. The existence of two subclasses of AT_1 receptor in rodents is established (Yoshida *et al.* 1992), but such a division in bovines has not been demonstrated.

Sequence analysis showed (Figure 5.8) that in fact these two anomalous clones did contain AT_1-derived sequence and the observed differences in restriction pattern arose from sequence artefacts generated during PCR amplification. They were therefore not analysed any further. The discrepancies observed in the 5′ sequences of these two clones could explain the differences observed in fragment size after restriction digests : approximately 50 and 80 additional bases respectively were present in $pAT_1.43$ and 50. The presence of these extra bases would have led to larger bands being observed upon restriction mapping. This does not, however, explain the absence of the MunI site in clone $pAT_1.43$. This is probably due to a PCR single base incorporation error, making the site unrecognisable to the enzyme. The fact that this error occurred in only one clone makes this explanation more probable.

Clones $pAT_1.2C$ and 44 were selected as examples of AT_1 receptor clones for further sequence analysis. They were sequenced along their entire length, and comparison of the full sequences for $pAT_1.2C$ and 44 with the published sequence revealed a number of discrepancies summarised in Table 5.2. The single base substitutions found in clones $pAT_1.2C$ and 44 when compared to the published sequence are probably attributable to PCR

copying errors. These errors have occurred at a rate comparable to those reported for Taq polymerase (8.0×10^{-6} mutations/bp/duplication; Cline *et al.*, 1996). As Taq polymerase has no proof-reading ability, they could have been averted by the use of a proof-reading polymerase : proof-reading capability can raise the fidelity of DNA replication up to 100-fold (Kunkel, 1992). However, the enzyme initially tested in PCR optimisation, ULTma, proved unable to amplify the AT_1 receptor coding region under any of the conditions tested, possibly due to its strong 3'-5' exonuclease activity and consequent lack of processivity. It is also possible that the misincorporation may have been a sequence-specific problem. Both pAT_1.2C and 44 contain a single misincorporated base (81 bases into AT_1 sequence for 2C; 168 bases into AT_1 sequence for 44) early into the amplimer length. If ULTma had progressed to that point, found a misincorporated base and repaired it, and then been unable to re-start polymerisation, successful amplification could not have occurred. This might possibly explain, in part, the difficulty experienced with this enzyme. Another possibility is that these single base substitutions could also be due to the presence of allelic or strain variants in AT_1 receptor sequences.

Table 5.2 shows two areas in each clone that have been incompletely resolved. Both strands had been sequenced along the length of the clones, and for the majority of the sequence had yielded only small compressions resolvable by use of dITP as a dGTP analogue. However, in these two areas, even use of dITP was not sufficient to resolve the compressions resulting from the C-G-richness of the region. Due to constraints of time, no further attention was given to this problem. Further sequence analysis, perhaps using alternative primers or another dGTP analogue (such as 7-deaza-dGTP) would, however, be the next step in obtaining full sequence for the clones along both strands. However, the degree of homology obtained with the published sequence, even with the unresolved areas, was 98.9% for pAT_1.2C and 98.4% for pAT_1.44. This was sufficient for a probe generated from these

clones to successfully hybridise to AT_1 sequence under the chosen hybridisation conditions as shown in Figure 2.4.

The sequence data obtained to date is sufficient to show that the receptor sequenced is of the AT_1 class and not the AT_2 subtype. This second subtype shares only 36% homology with the type 1 receptor, making it extremely unlikely that it could have been amplified using the primers chosen. It can also be concluded that two subtypes of AT_1 receptors have not been isolated in this cloning experiment; this possibility having been briefly considered when examining the restriction mapping anomalies. To date, no large mammals have been found to have AT_1 receptor subtypes.

The main aim of this chapter was to produce a clone of the bovine adrenal AT_1 receptor. Successful amplification of the bovine adrenal AT_1 receptor coding region and insertion of this sequence into a cloning vector pSP72poly4 yielded 12 clones, 10 of which were found to contain AT_1 sequence by restriction mapping. Two of these were fully sequenced and showed over 98% homology with the published sequence. These clones were then used to generate DNA for use as a probe for AT_1 mRNA in Northern blots. When hybridised to Northern blots of bovine adrenocortical cell RNA, a transcript of 3.3kb was detected (Figure 2.4), as shown by Sasaki *et al.* (1991). (The probe generated by random priming gave a slightly less sensitive signal than that generated by asymmetric PCR. However, the increased simplicity of the 'Vogelstein' method, coupled with only a slight loss of signal, led us to adopt this method for routine preparation of probes). This furnishes further evidence that the clones generated and sequenced in this chapter were in fact bovine adrenal AT_1 receptor clones.

Chapter 6 : Regulation of bovine adrenocortical AT_1 receptor mRNA expression in cultured bovine adrenocortical cells

6.1 Introduction

Angiotensin II receptors represent an important point of control of AII action. An increase in number or affinity of the receptors could lead to an increased response to the hormone and vice versa. Indeed, it has already been observed that sodium restriction increases AII receptors in the adrenal cortex, and that this sensitises the response of this tissue to AII (Aguilera *et al.* 1980). It has further been discussed in section 1.5 that alterations in the activity of the renin-angiotensin system can have important pathophysiological implications; alterations in normal receptor regulation, as one component of this system, could therefore also be important.

The adrenal cortex is one of the prime target organs for angiotensin II and adrenocortical AII receptors have been extensively studied in many species. We chose to examine the regulation of AII receptor mRNA in primary cultures of bovine adrenal zona fasciculata cells. Both bovine zona glomerulosa and zona fasciculata cells in culture respond to AII stimulation in the same manner as human adrenal cells (Naville *et al.* 1993). Although it is accepted that the zona glomerulosa is the most important zone for AII action in the adrenal cortex, the receptors in the inner zone also induce a steroidogenic response to AII (Clyne *et al.* 1993; Ouali *et al.* 1993; Lebrethon *et al.* 1994). Furthermore, they are pharmacologically identical to those in the zona glomerulosa (see Chapter 4) and they utilise the same signal transduction systems (Ouali *et al.* 1992; Clyne *et al.* 1993). Due to the large numbers of cells required to carry out the planned experiments, use of zona glomerulosa cells would have limited the studies as the yield of cells from

these preparations was usually only a tenth of that from inner zone preparations. Therefore the inner zone cells were selected as a model system in these studies rather than outer zone cells.

The regulation of receptors can be studied at more than one level. Most commonly, the levels of receptor protein (by radioligand binding or Western blotting) or mRNA (by Northern blotting, RNAse protection or RT-PCR) are analysed. We chose to analyse the steady-state levels of AT_1 receptor mRNA by Northern blotting. Changes in mRNA levels would be expected to occur sooner after treatment with an agonist than changes in receptor protein levels, and this therefore allows a more immediate determination of whether the levels are being increased or decreased. Analysis of mRNA also precludes interference with measurement of receptor protein levels (by radioligand binding) due to internalisation of the AII receptor. Northern blot analysis is the simplest method of analysing steady-state mRNA levels and was therefore the method of choice in this study.

As the receptor subtype mediating the physiological actions of AII in the zf and zg of the bovine adrenal cortex is known to be the AT_1 subtype (Clyne *et al.* 1993), it was the regulation of AT_1 receptor mRNA levels which were studied.

The regulation of AT_1 receptors has been widely studied. It has been shown that AII can downregulate its own binding sites (i.e. receptor number) in bovine adrenocortical cells (Penhoat *et al.* 1988), and that this can be mimicked by stimulating the IP_3/Ca^{2+} signal transduction system with a combination of phorbol ester and calcium ionophore. ACTH and other cAMP stimulators and analogues also decrease AII receptor number in cultured bovine adrenocortical cells (Penhoat *et al.* 1988). While this work has revealed a similar regulatory pattern to the few studies in human adrenal primary cell cultures (Naville *et al.* 1993), it has only encompassed studies of AII binding, and not the expression of the AT_1 receptor mRNA. Recently, Bird *et al.* have demonstrated that the AT_1 receptor in the human adrenocortical cell line, H295R, is regulated in a similar pattern to previous

studies in both human and bovine adrenocortical cell primary cultures (Penhoat *et al.* 1988; Naville *et al.* 1993), and have shown that changes in AT_1 receptor mRNA levels precede the observed changes in AII binding sites (Bird *et al.* 1995a). We therefore wished to study the regulation of the AT_1 receptor at the mRNA level in primary cultures of bovine adrenocortical cells, in order to determine whether the previously documented changes in AII receptor number were correlated with any changes in AT_1 receptor mRNA levels.

It was wished to examine effects on the AT_1 receptor mRNA level induced by AII itself, and by direct stimulation of the signal transduction pathway it stimulates, i.e. inositol phosphate/calcium. The effects on AT_1 mRNA levels of activation of both the cyclic AMP and tyrosine kinase signal transduction systems, which were both known to affect AII receptor number in bovine zfr cells (Penhoat *et al.* 1988; Lebrethon *et al.* 1994), were also examined in this study.

Agonists were chosen that could have effects on the receptor physiologically, or that directly activated adrenocortical second messengers. Therefore both AII and ACTH were studied, in addition to direct activators of the IP_3/Ca^{2+} (phorbol ester and calcium ionophore) and cAMP (8Br-cAMP) signal transduction systems. IGF-1, known to activate tyrosine kinase, has also been demonstrated to be active in the adrenal cortex (Weber *et al.* 1994) and was studied for that reason. Potassium is important in the control of the renin-angiotensin system, and stimulates steroidogenesis in both zones of the human adrenal cortex in culture (Laird *et al.* 1991). Thus it was also considered as a possible regulatory influence on AT_1 mRNA levels.

Finally, it was wished to consider the effects of adrenocortical steroids themselves on AT_1 receptors. Two were studied : aldosterone and cortisol. Both are produced by the action of AII on its receptor and are therefore possible candidates for a product feedback type of regulation. Physiologically, zona fasciculata cells are exposed to high concentrations of cortisol (Crivello *et al.* 1983) and might also be exposed to high

concentrations of aldosterone before it reaches the adrenocortical capillary network.

The aims of this chapter were, therefore, to examine the regulation of bovine adrenocortical AT_1 receptor mRNA levels, and also the possibility that the products of AT_1 receptor stimulation in the two zones of the adrenal cortex participate in a feedback system. A consideration is also given to whether the agents used to regulate the receptor raise basal steroid output in these cells and whether this contributes to the observed regulation.

The experiments in this chapter were designed to examine the regulation of steady-state levels of AT_1 receptor mRNA. Preliminary studies to determine the time-course and dose-dependency of the effects of given agonists were carried out twice. Three further experiments were then performed to analyse quantitatively the effects of these agonists on AT_1 receptor mRNA levels at times and concentrations shown previously to be the most effective. Although the hybridisation procedure in Northern blotting is semi-quantitative, the changes in levels could be quantitated by comparing AT_1 receptor mRNA levels in treated cells to basal or control samples (i.e. untreated cells) within an experiment. The quantitation of signal from the hybridised blots on a phosphorimager was, however, fully quantitative. To correct for loading variation on the blots, AT_1 mRNA levels were normalised to levels of β-actin mRNA before comparison with the basal levels. The quantitated data is therefore presented as a percentage of basal levels of the ratio AT_1 receptor mRNA : β-actin mRNA. Statistical analysis was performed using Student's t-test, with significance inferred when $p<0.05$.

6.2 Results

6.2.1 Levels of bovine adrenal AT_1 receptor mRNA in culture.

Before examining the regulation of AT_1 receptor mRNA levels, it was wished to determine how these levels varied basally during the first few days of culture. Bovine zona fasciculata/reticularis cells were isolated and cultured as described in Chapter 2 (2.2.2.2). The cells were plated at a density of 5 $x10^6$ cells per $25cm^2$ flask (as in 2.2.2.9); one flask of cells was used per experimental point. After 24 hours in culture, RNA was isolated from the cells in one flask. The medium was changed in the remaining flasks. On the following morning, RNA was extracted from another flask and the remaining cells were serum deprived. Six hours later, another flask was removed and the RNA isolated. At 24 and 48 hours following the 6h serum deprivation, RNA was isolated from a further flask. RNA from all samples was then run on a Northern blot, which was probed for both AT_1 and β-actin mRNA. Figure 6.1 shows the autoradiograph from this experiment, and Table 6.1 shows the quantitation of the signal from this autoradiograph.

For the first two days in culture the AT_1 receptor mRNA level appeared to be undergoing a recovery period : its expression increased to a constant level by the second day after isolation. Between days 2 and 4 in culture, there was very little variation in expression of the receptor mRNA. The increase observed in AT_1 mRNA levels during the first two days in culture was also observed for β-actin; this possibly reflects a general recovery after isolation. These results show that AT_1 receptor mRNA levels do not change appreciably during the time period 48-96h after isolation. This period was therefore chosen for experimentation, and any changes appearing during experimentation can thus be attributed to the agonists used.

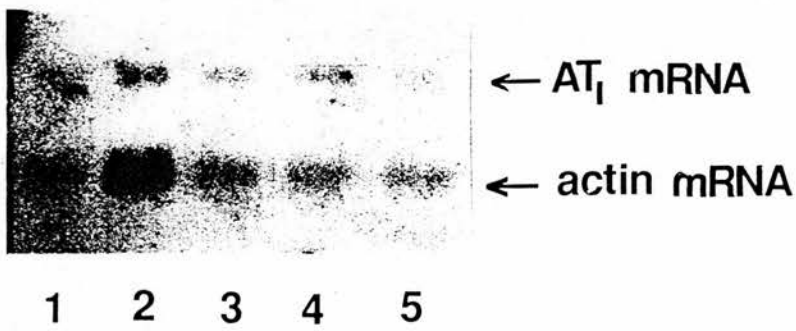


Figure 6.1 Basal levels of bovine adrenal AT_1 receptor mRNA expression in culture

Bovine zfr cells were cultured as described in 2.2.2.2. 40 hours after isolation the cells were serum deprived for 6h. Fresh serum-free medium was then added and changed every day. Samples of RNA were taken throughout the culture period. RNA was harvested after 1 day in culture (t=24h, lane 1), 1 day plus overnight in culture (t=40h, lane 2), two days in culture including 6 hours' serum deprivation (t=46h, lane 3), and 3 and 4 days in culture, serum deprived (t=60h, lane 4; t=84h, lane 5). Both AT_1 and β-actin mRNAs are shown on this Northern blot autoradiograph. Levels of both species of RNA increased during the first two days in culture and remained constant thereafter. β-actin was more highly expressed in the bovine adrenal cell than was AT_1 receptor mRNA.

Day after isolation	AT_1 mRNA (normalised to β-actin)
1 (t=24h)	0.32
1 + overnight (t=40h)	0.47
2 (t=46h) *	0.75
3 (t=60h) *	0.75
4 (t=84h) *	0.72

Table 6.1 : Levels of AT_1 mRNA expression during culture

Bovine zfr cells were cultured and sampled during the course of the experiment described in 6.2.1 in order to assess the level of expression of the AT_1 receptor mRNA. Data (expressed as arbitrary units) obtained from the Northern blot shown in Figure 6.1. (* = serum-deprived).

6.2.2 Effect of AII on the expression of the AT_1 receptor

Zfr cells were treated with AII in order to investigate the homologous regulation of AT_1 receptor mRNA levels. The concentration previously shown (Figure 3.3) to elicit a maximal steroidogenic response from zg cells, 10nM AII, was used to study the time course of regulation. Cultures of bovine zfr cells were treated with 10nM AII for periods of between 4 and 48 hours and Figure 6.2 shows a representative Northern blot autoradiograph from such an experiment. There was a clear decrease of the receptor mRNA by 8 hours, which recovered to basal levels by 24 to 48 hours.

The concentration-response of the effect of AII was next studied. Zfr cells were treated with doses of AII ranging from 0.1nM to 100nM for 6 hours. A clear effect on the level of the AT_1 receptor mRNA was seen at 1nM, with a lesser effect at 0.1nM. 10 and 100nM AII effectively abolished AT_1 receptor mRNA expression (Figure 6.3).

A quantitative assessment of this effect was then obtained from triplicate experiments. Bovine zfr cells were cultured as described and treated with 1 and 10nM AII for 6 hours, and 10nM AII for 48 hours. Figure 6.4 shows a bar chart combining the results from all three experiments, while figure 6.5 shows an autoradiograph of a representative experiment. The most significant effect resulted from a treatment of 10nM AII for 6 hours, which decreased AT_1 receptor mRNA levels by 84% compared to control levels ($p<0.005$). This effect was concentration-dependent, as 1nM AII produced a smaller decrease of 64% ($p<0.005$). A chronic exposure to AII produced only a slight, non-significant decrease of 21%, showing that the receptor levels return to almost control levels on prolonged exposure to AII.

6.2.3 Effect of stimulants of the IP_3/Ca^{2+} signal transduction system

Cultures of bovine adrenal zfr cells were exposed to two compounds : a phorbol ester, phorbol myristate-12-acetate (PMA), was used to directly stimulate protein kinase C, and a calcium ionophore, A23187, was used to

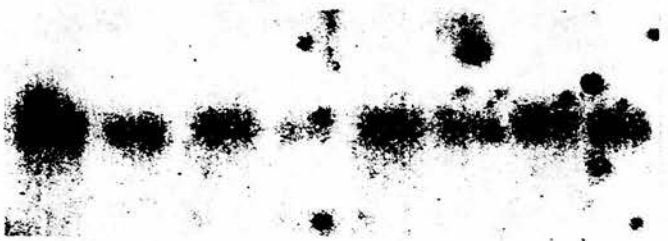
Effect of AII on the levels of AT_1 mRNA

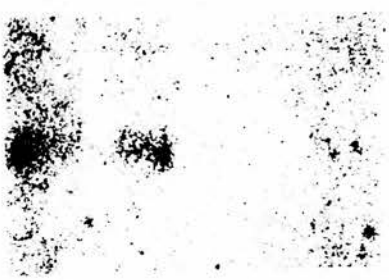
Figure 6.2

The time course of AII's effect on AT_1 receptor mRNA levels was investigated. Bovine zfr cells were cultured for two days, serum deprived for 6 hours and then treated with 10nM AII for, as shown on the blot left to right, 8, 16, 24 and 48 hours (lanes 2,4,6,8). The corresponding control (basal) samples are also shown (lanes 1,3,5,7). A decrease in AT_1 receptor mRNA levels was apparent and maximal after 8 hours.

Figure 6.3

The concentration-dependency of the effect of AII on its receptor mRNA expression was also determined. Bovine adrenal zfr cells were cultured as above and incubated for 6 hours with various concentrations of AII. Left to right the lanes are : control, 0.1nM, 1nM, and 10nM AII. A decrease in AT_1 receptor mRNA levels was apparent at 1nM.


1 2 3 4 5 6 7 8


1 2 3 4

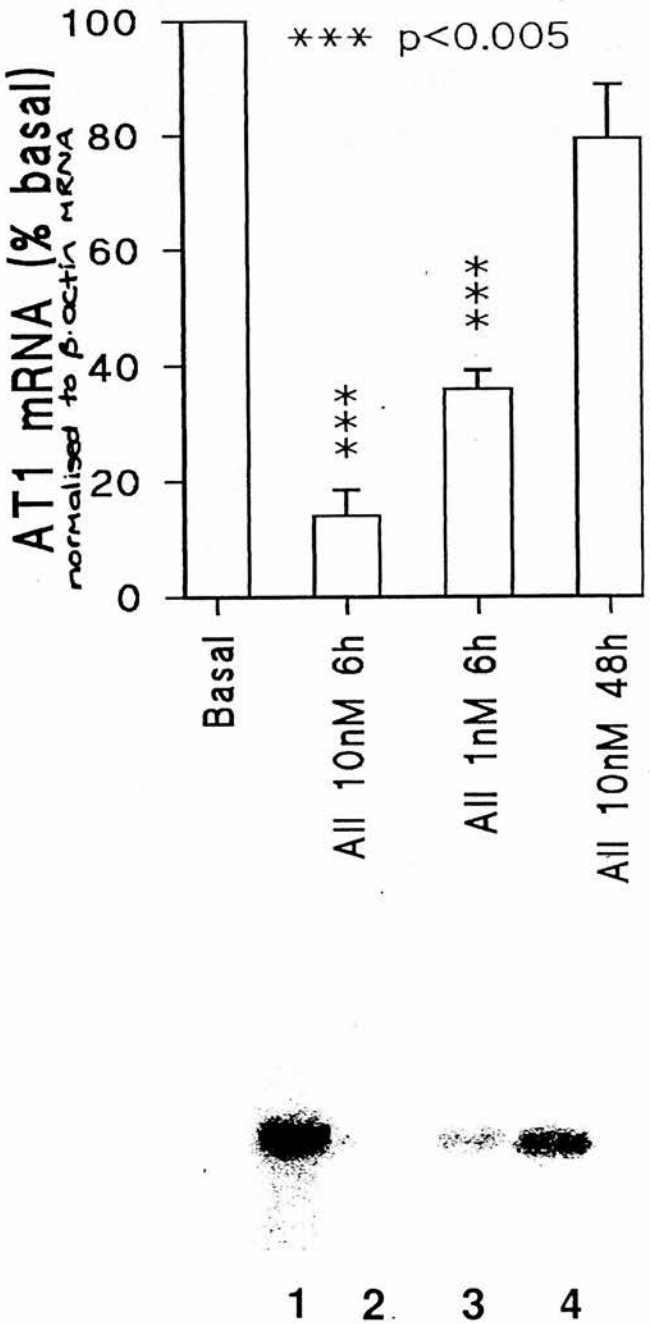
Quantitative effect of AII on AT_1 receptor mRNA levels

Figure 6.4

The effect of AII on AT_1 receptor mRNA was quantitated as described in 6.1. Bovine zfr cells were cultured for 2 days and then serum deprived for 6h (as in 2.2.2.9) before being treated with 1 and 10nM AII for 6h and 10nM AII for 48h. The percentage of AT_1 receptor mRNA (normalised to β-actin) compared to basal levels is shown as the mean +/- s.e.m. of three experiments. A significant decrease ($p<0.005$) in AT_1 mRNA was seen after exposure to 1 or 10nM AII for 6h, with recovery occurring after 48h treatment with 10nM AII.

Figure 6.5

A representative Northern blot autoradiograph from the experiments described above. Lanes 1-4 correspond to the bars left to right in Figure 6.4.


AT1 mRNA (% basal)
normalised to β-actin mRNA
100
80
60
40
20
0
*** p<0.005
***
***
Basal
All 10nM 6h
All 1nM 6h
All 10nM 48h
1
2
3
4

stimulate calcium influx into the cells. The two compounds were used singly and in combination at concentrations of 1μM.

The result of a representative experiment to determine the time-course for the effects of these two compounds is shown in Figure 6.6. PMA and A23187 yielded very similar effects when applied separately, and more pronounced effects when applied in combination. A dramatic decrease in AT_1 receptor mRNA levels was apparent by 6h treatment, which recovered to varying extents by 48h for the single treatments and not at all for the combined treatment.

A dose-response experiment was also carried out (Figure 6.7). The cells were treated for 48h with concentrations ranging from 0.1nM to 1μM of PMA, A23187 or a combination of the two (A+P). The highest concentration for the single treatments produced a decrease of the levels of AT_1 receptor mRNA, but most of the lower concentrations were without effect. A23187 was effective at decreasing AT_1 receptor mRNA levels at lower concentrations than was PMA; the combination was again most effective as a decrease in AT_1 receptor mRNA levels was apparent at concentrations as low as 10nM.

For quantitation of this effect, time-points of 6 and 48 hours and a concentration of 1μM PMA and A23187 were chosen, as these gave maximal effects, and allowed a comparison to be made between acute and chronic exposure. Three experiments were carried out and are summarised in Figure 6.8, whilst a representative autoradiograph from one of these experiments is shown in Figure 6.9. Of the two agonists, PMA was the most effective, causing a decrease in AT_1 mRNA levels by 87% ($p<0.01$) after a 6 hour, 1μM treatment. A23187 caused a decrease of 67% after the same time ($p<0.01$), and the two treatments combined decreased the AT_1 mRNA levels by 91% after 6 hours ($p<0.005$). As can be seen in Figure 6.6, β-actin mRNA levels were not affected by these treatments. Chronic exposure (48 hours) to 1μM PMA produced a decrease of 25% in AT_1 receptor mRNA levels, comparable to that induced by 10nM AII. However, 1μM A23187 appeared to produce an

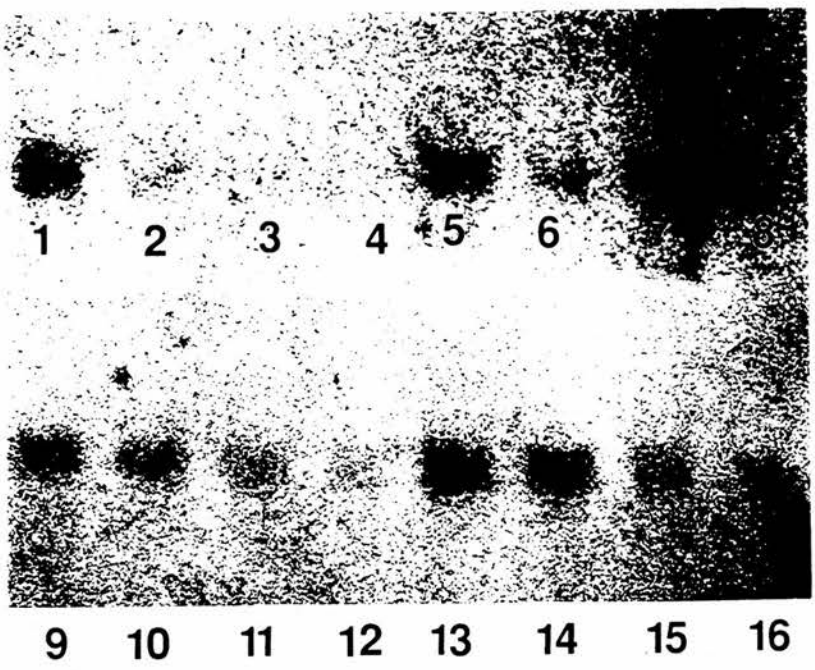
Effect of activation of the IP_3/Ca^{2+} signal transduction system on AT_1 receptor mRNA steady state levels

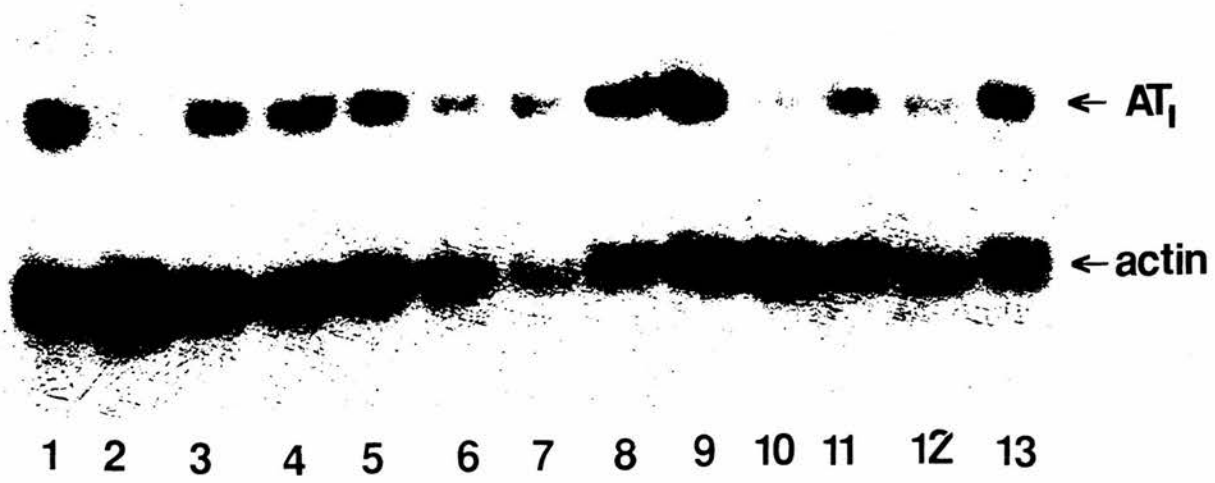
Figure 6.6

Levels of AT_1 receptor mRNA after treatment of cultured bovine zfr cells with phorbol ester (PMA), calcium ionophore (A23187), and the two in combination (A+P), all at 1μM, to determine the time course of their effect on AT_1 receptor mRNA levels. Bovine zfr cells were cultured as previously described, serum deprived for 6h and exposed to these agents for 6, 18, 24 and 48h, as shown on this representative Northern blot autoradiograph. Control (basal) samples are included for each time point. Lanes 1,5,9,13: control; 2,6,10,14: 1μM PMA; 3,7,11,15: 1μM A23187; 4,8,12,16: 1μM A+P.

Figure 6.7

Representative experiment to determine the dose-dependency of the effects shown above. Bovine zfr cells were cultured as described previously, incubated for 48 hours with 1nM, 10nM, 100nM and 1μM PMA, A23187 or A+P. A control (basal) RNA sample is run in lane 1; from left to right in, decreasing concentrations, are PMA (lanes 2-5), A23187 (lanes 6-9) and A+P (lanes 10-13). β-actin mRNA levels are also shown to illustrate that the treatments used had no effect on its mRNA levels.


1
2
3
4
5
6
9
10
11
12
13
14
15
16


← AT1
←actin
1
2
3
4
5
6
7
8
9
10
11
12
13

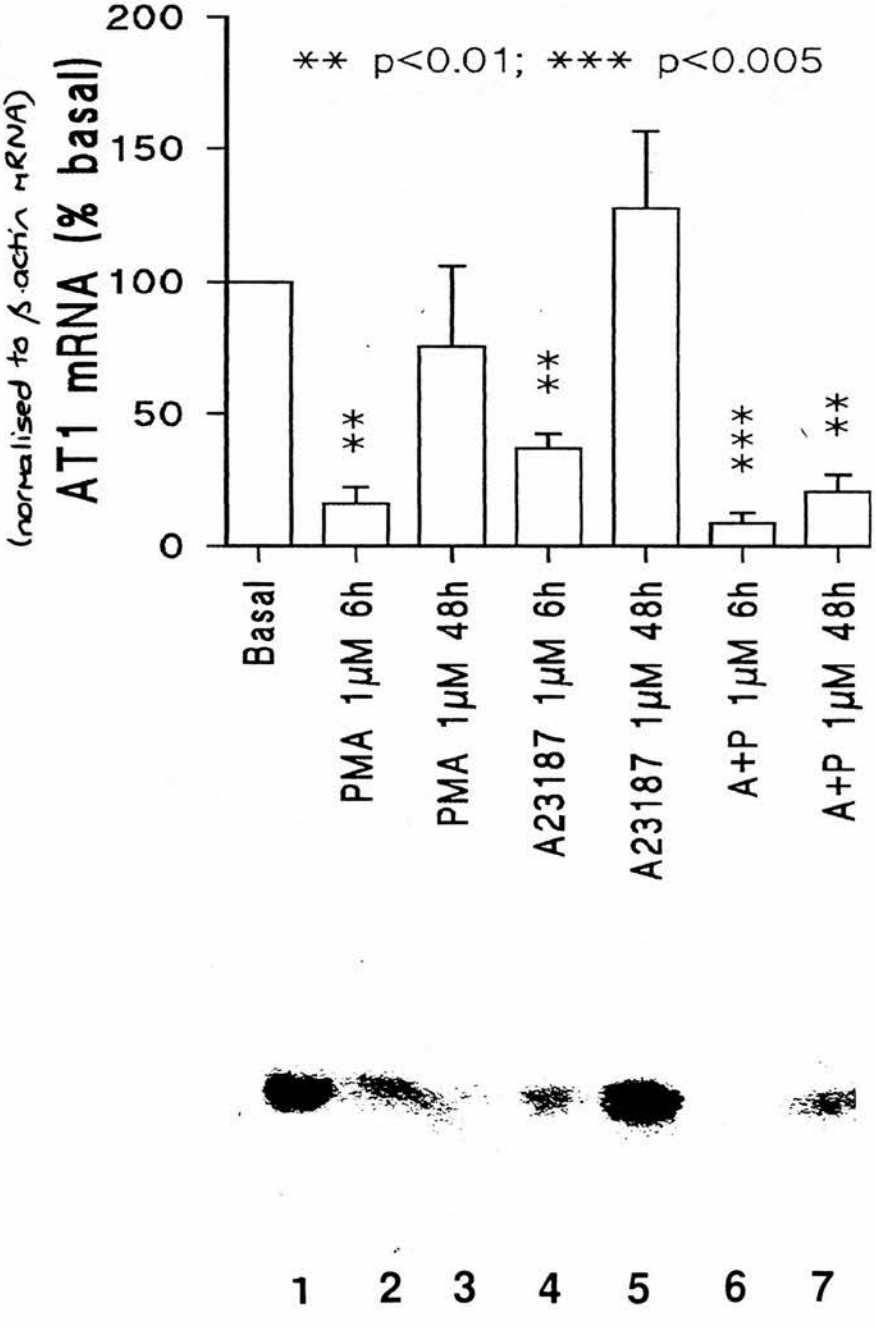
Quantitative effects of stimulation of the IP_3/Ca^{2+} signal transduction system on AT_1 receptor mRNA levels.

Figure 6.8

The percentage of basal levels of AT_1 receptor mRNA were determined following treatment of bovine zfr cells with 1μM phorbol ester (PMA) and calcium ionophore (A23187), either singly or in combination (A+P) for 6 and 48h. The mean and standard error of AT_1 receptor mRNA levels from three experiments are shown.

Figure 6.9

A Northern blot autoradiograph from a representative experiment of those described above. The lanes 1-7 are aligned to the bars corresponding to the relevant treatments in Figure 6.8.


(normalised to β-actin mRNA)
AT1 mRNA (% basal)
200
150
100
50
0
** p<0.01; *** p<0.005
**
**
**
***
Basal
PMA 1μM 6h
PMA 1μM 48h
A23187 1μM 6h
A23187 1μM 48h
A+P 1μM 6h
A+P 1μM 48h
1
2
3
4
5
6
7

increase, giving rise to AT_1 receptor mRNA levels 128% of control. The combined treatment, as expected from the qualitative experiments, still depressed the level of AT_1 receptor mRNA after 48 hours, although a slight recovery had taken place, to 21% of control ($p<0.01$ vs. basal).

6.2.4 Effect of stimulants of the cyclic AMP signal transduction system

To examine the effects of stimulating the cyclic AMP/protein kinase A signal transduction system, cultured bovine zfr cells were treated with ACTH, a physiological activator of the system, and also 8-Br-cAMP, a cAMP analogue.

A representative time course for the effect of 1nM ACTH and 0.1mM 8-Br-cAMP on the levels of AT_1 receptor mRNA in bovine zfr cells is shown in figures 6.10a and 6.10b. Both compounds produced a decrease in AT_1 receptor mRNA levels; this effect first became apparent at 8 hours for 8-Br-cAMP (Figure 6.10a), but at 48h for ACTH (Figure 6.10b), AT_1 receptor mRNA levels reaching a minimum level by 48 hours of treatment in both cases.

To determine the dose-dependency of this decrease, bovine zfr cells were treated over a range of concentrations with one or other of the agonists for 48 hours (Figure 6.11). A concentration as low as 1μM 8-Br-cAMP produced a noticeable effect on AT_1 receptor mRNA levels, with a maximal effect at 0.1mM. ACTH produced a decrease even at 0.01nM, with a maximal effect at 1nM.

To quantitate the observed effects on AT_1 receptor mRNA levels, bovine zfr cells were exposed to 0.1mM 8-Br-cAMP and 1nM ACTH for 6 and 48 hours, to reveal any difference between acute and chronic exposure. These experiments are shown in Figures 6.12 and 6.13, the former showing the combined results from three experiments plotted as a bar chart, and the latter a representative autoradiograph. Both agonists produced a greater decrease in AT_1 receptor mRNA levels after 48 hours : to 16% of control

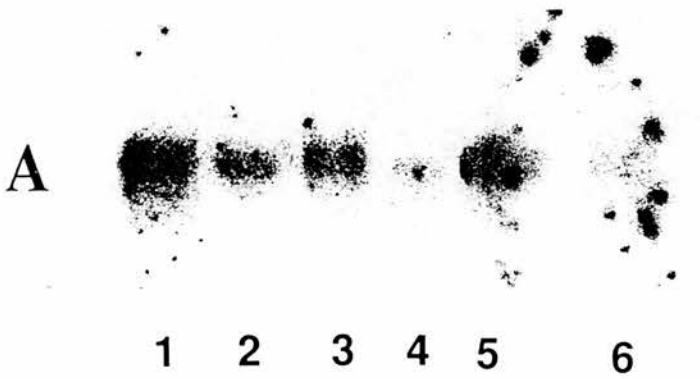
Effect of activation of the cyclic AMP signal transduction system on AT_1 receptor mRNA levels

Figure 6.10

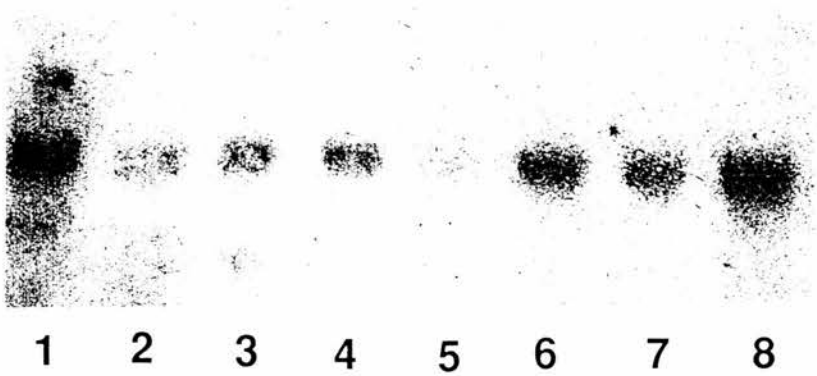
This representative experiment assessed the time course of the effects of stimulation of the cyclic AMP second messenger pathway on levels of AT_1 receptor mRNA. Bovine zfr cells were cultured for two days, serum deprived for 6h and then incubated with either 0.1mM 8-Br-cAMP for 8, 24 and 48h (Figure 6.10a) or 1nM ACTH (Figure 6.10b) for 6, 18, 24 or 48 hours (lanes 2,4,6,(8) in increasing time). Control samples are also shown (lanes 1,3,5,(7)).

Figure 6.11

The dose-dependency of the response of AT_1 receptor mRNA levels to activation of the cAMP signal transduction system was then determined. Bovine zfr cells were incubated with 0·1, 1, 10, 100μM 8-Br-cAMP and 0·01, 0·1, 1nM ACTH for 48 hours. A control sample is shown in lane 1 and then left to right are ACTH in decreasing concentrations (i.e. 1nM to 0.01 nM), and 8-Br-cAMP also in decreasing concentrations.


A
1 2 3 4 5 6


B
1 2 3 4 5 6 7 8


1 2 3 4 5 6 7 8

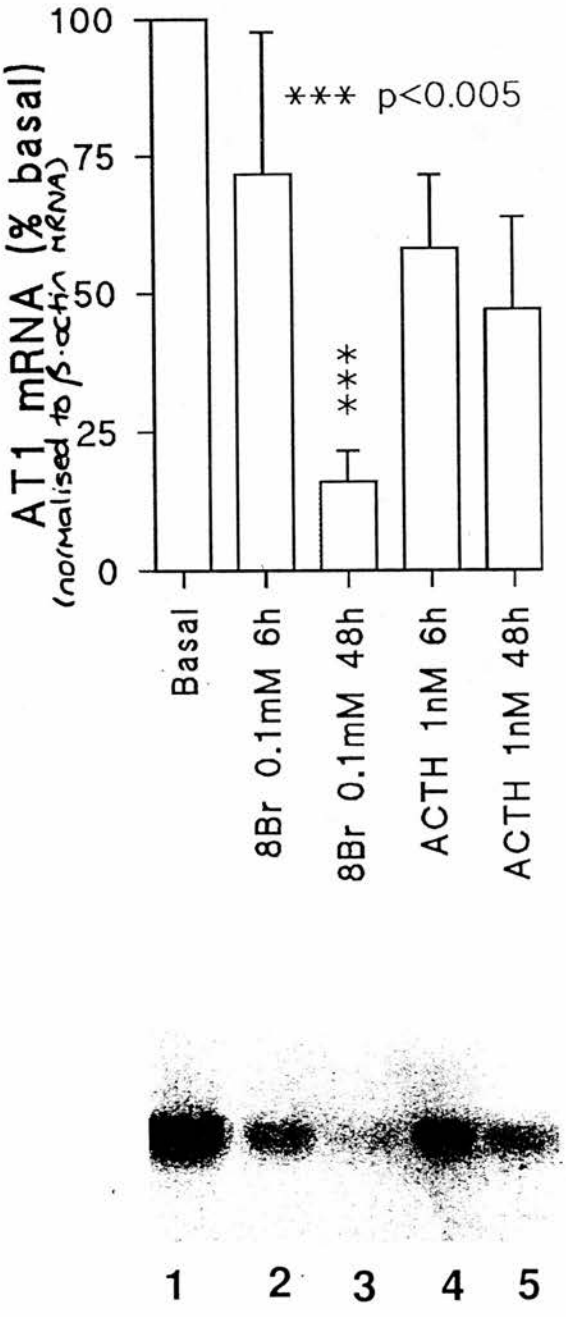
Quantitative effect of stimulation of the cyclic AMP signal transduction system on AT_1 receptor mRNA levels.

Figure 6.12

Changes in AT_1 receptor mRNA levels determined quantitatively following stimulation of the cyclic AMP second messenger system by either the physiological ligand, ACTH, or the cAMP analogue, 8-Br-cAMP. Bovine zfr cells were cultured as described previously and treated with 0.1mM 8Br-cAMP or 1nM ACTH for 6 or 48h. The mean and standard error of AT_1 receptor mRNA levels from three experiments are shown.

Figure 6.13

A Northern blot autoradiograph from a representative experiment of the three described above. The lanes 1-5 are aligned left to right with the corresponding treatments detailed in Figure 6.12.


AT1 mRNA (% basal)
(normalised to β-actin mRNA)
100
75
50
25
0
*** p<0.005
***
Basal
8Br 0.1mM 6h
8Br 0.1mM 48h
ACTH 1nM 6h
ACTH 1nM 48h
1
2
3
4
5

levels for 8-Br-cAMP ($p<0.005$) as opposed to 72% after 6 hours, while treatment with 1nM ACTH tended to decrease receptor mRNA levels to AT_1 55% and 47% after 6 and 48h respectively. Only the chronic (48h) treatment with 8Br-cAMP yielded a statistically significant decrease in AT_1 receptor mRNA levels.

6.2.5 : Effects of stimulants of other signal transduction systems on AT_1 receptor mRNA steady state levels in bovine zfr cells

A representative time-course for the effects of the tyrosine kinase agonist IGF-1 and potassium on the levels of AT_1 receptor mRNA is shown in figure 6.14. Bovine zfr cells were cultured for 2 days, serum deprived for 6h and then treated with 10ng/mL IGF-1 and 12mM potassium for 8, 16, 24, and 48h. IGF-1 at 10ng/mL initially induced a decrease in AT_1 receptor mRNA levels, as seen after 8 hours incubation. However, after longer treatment, this was reversed and a distinct increase became apparent. Potassium (12mM) also increased AT_1 receptor mRNA levels, and this effect was apparent after 16 hours incubation, reaching a maximum by 48 hours treatment (figure 6.14).

The dose-dependency of these effects was then studied. Cells were treated with a range of concentrations of the two agonists for 48 hours and a representative autoradiograph can be seen in Figure 6.15. 10ng/mL IGF-1 induced the highest increase in AT_1 receptor mRNA levels, with 5ng/mL having little effect and the lowest concentration none at all on AT_1 receptor mRNA levels. Potassium, however, showed an unusual dose-response. 8mM potassium had no effect on the levels of AT_1 receptor mRNA, 12mM, as previously seen, increased the mRNA levels, and 16mM appeared to cause a decrease in AT_1 receptor mRNA levels.

The effect of 10ng/mL IGF-1 and 12mM potassium were further investigated by quantitative analysis of three further Northern blot experiments. Bovine zfr cells were cultured as previously described and treated with 10ng/mL IGF-1, 12 or 16mM K^+, for 6 and 48h. The mean (+/-

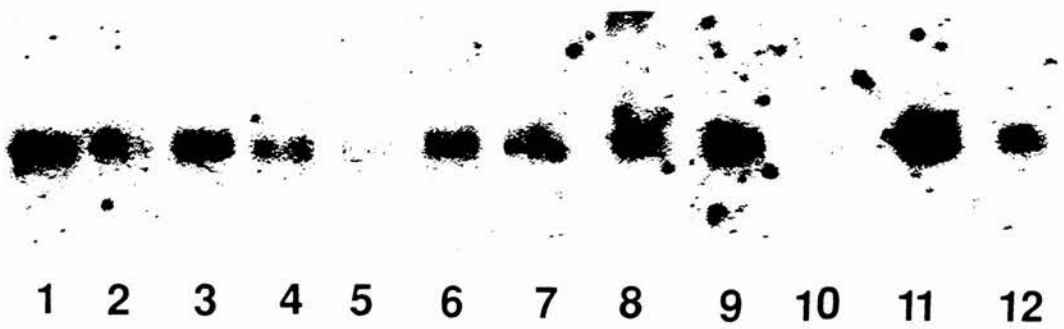
Effect of activating other signal transduction systems on AT_1 receptor mRNA levels

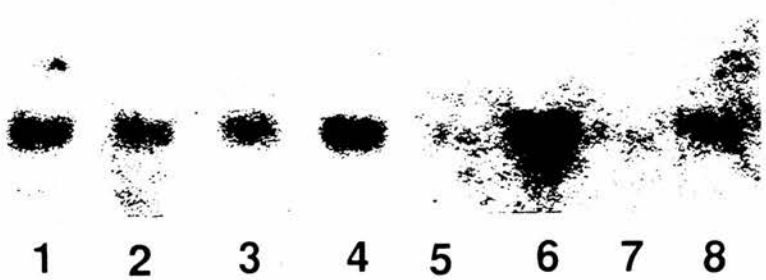
Figure 6.14

Effects of IGF-1 - mediated activation of tyrosine kinases and potassium on levels of AT_1 receptor mRNA in cultured bovine zfr cells. Cells were cultured for 2 days and serum deprived for 6h, then treated with 10ng/mL IGF-1 or 12mM potassium for 8, 16, 24 and 48 hours; control samples were also taken at each time point. Across this Northern blot autoradiograph left to right run the time points in increasing duration for control (lanes 1,4,7,10), IGF-1 (lanes 2,5,8,11) and potassium (lanes 3,6,9,12).

Figure 6.15

The dose-dependency of the above affects was then determined. Bovine zfr cells were cultured for two days, serum deprived and incubated for 48 hours with 1, 5 and 10ng/mL IGF-1, and 8, 12, 16, and 20mM potassium. A control sample was also run; this appears in lane 1, the IGF-1 samples in lanes 2-4 and the potassium samples in lanes 5-8, in order of increasing concentration.


1 2 3 4 5 6 7 8 9 10 11 12


1 2 3 4 5 6 7 8

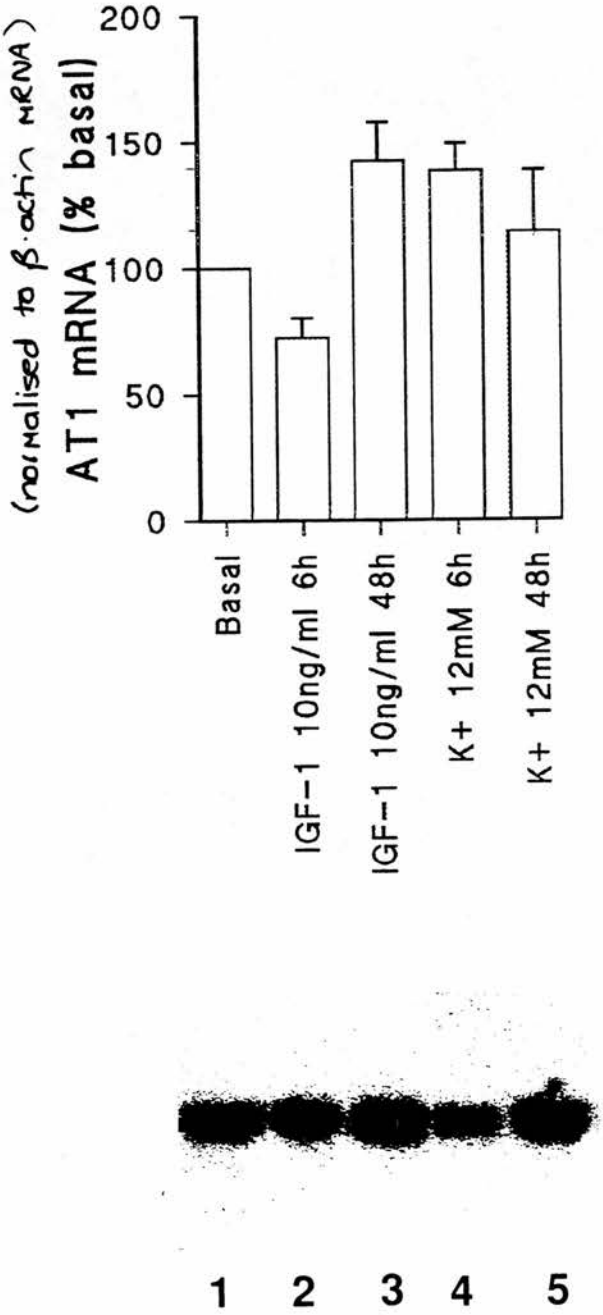
Quantitative effects of IGF-1 and K^+ on AT_1 receptor mRNA levels

Figure 6.17

The effects of IGF-1 and K^+ on expression of AT_1 mRNA were then quantitated. Bovine adrenocortical cells were cultured as previously described and treated with 10ng/mL IGF-1 or 12mM K^+ for 6 or 48 hours; the bars show the mean +/- s.e.m of AT_1 receptor mRNA levels for three separate experiments.

Figure 6.18

A representative autoradiograph from a Northern blot of one of the above experiments. The numbers on the lanes left to right correspond to the agonists shown left to right in Figure 6.17.


(normalised to β-actin mRNA)
AT1 mRNA (% basal)
200
150
100
50
0
Basal
IGF-1 10ng/ml 6h
IGF-1 10ng/ml 48h
K+ 12mM 6h
K+ 12mM 48h
1
2
3
4
5

K^+ Concentration (mM)	Incubation time (hours)	AT_1 mRNA (% basal) mean +/- sem
12	6	138 +/- 10
12	48	114 +/- 24
16	48	83 +/- 12

Table 6.2 : Quantitative effects of potassium on expression of AT_1 receptor mRNA levels

Mean +/- sem (from three combined experiments) of AT_1 receptor mRNA levels in bovine zfr cells after treatment with potassium for the stated concentrations and times.

s.e.m) percentage of basal AT_1 receptor mRNA levels following these treatments can be seen in Figure 6.16, while a representative autoradiograph is shown in Figure 6.17. No significant increases in AT_1 receptor mRNA levels were observed due to the degree of variation between experiments, but a trend towards increasing levels by both agonists can be seen. A decrease of AT_1 mRNA levels by IGF-1 after 6 hours exposure was also clearly evident.

Table 6.2 shows the mean levels of AT_1 mRNA in bovine zfr cells treated with 12 and 16mM potassium, to illustrate the contrasting effects obtained with these two concentrations. Acute stimulation (6h) with 12mM potassium induced an increase AT_1 receptor mRNA levels, which decreased after chronic (48h) treatment. Use of the higher concentration of potassium (16mM) tended to decrease the levels of AT_1 receptor mRNA.

6.2.6 : Effect of adrenal steroids on levels of AT_1 receptor mRNA in bovine zfr cells

Bovine zfr cells were cultured as described in 2.2.1.1 and after 6h serum deprivation they were exposed to a range of concentrations of two adrenal steroids, cortisol and aldosterone, for 48 hours. The concentrations of steroids used were representative of their physiological levels in the adrenal cortex (Crivello *et al.* 1983). The result of these experiments is shown in a representative Northern blot autoradiograph in Figure 6.18. It can be seen that even at the highest concentration used (100nM), aldosterone had no visible effect on the levels of AT_1 receptor mRNA, and was therefore not studied further. 10μM cortisol, however, induced a clear decrease of the AT_1 receptor mRNA levels, which was also noticeable at 1μM.

The time course of the effect of cortisol on AT_1 receptor mRNA levels was next investigated. Bovine zfr cells were cultured as described previously and incubated for 6, 18, 24 and 48h with 10μM cortisol. A decrease in AT_1 receptor mRNA levels was first apparent as early as 6 hours and indeed was

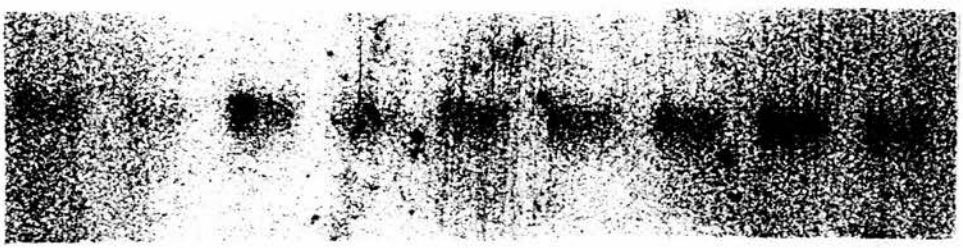
Effect of adrenal steroids on AT_1 receptor mRNA levels

Figure 6.18

Bovine zfr cells were cultured for two days, serum deprived and incubated for 48 hours with a range of concentrations of aldosterone and cortisol. This Northern blot autoradiograph shows a control sample run in lane 1; lanes 2-5 are cortisol samples (10μM, 1μM, 100nM and 10nM); lanes 6-9 are aldosterone samples (100nM, 10nM, 1nM and 0.1nM).

Figure 6.19

Bovine zfr cells were incubated with 10μM cortisol for 6, 24 and 48 hours toinvstigate the time course of cortisol's effect on AT_1 receptor mRNA levels. Control samples are shown in lanes 1, 3, and 5, and the treated samples in lanes 2, 4, and 6 in increasing duration of incubation.


1 2 3 4 5 6 7 8 9


1 2 3 4 5 6

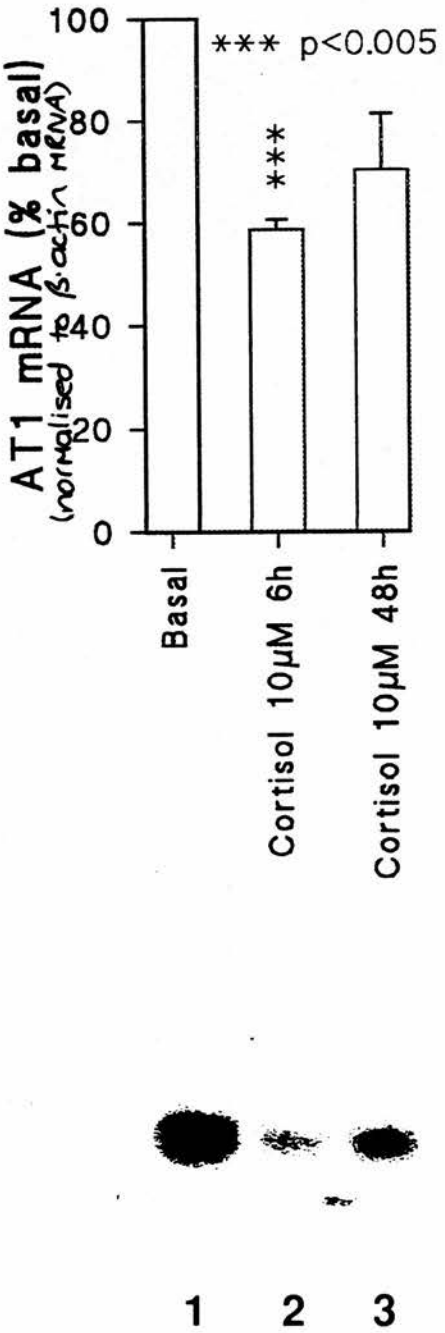
Quantitative effect of cortisol on levels of AT_1 receptor mRNA.

Figure 6.20

Levels of AT_1 receptor mRNA relative to basal expression after exposure of bovine zfr cells to 10μM cortisol for 6 and 48h. These data, expressed as the mean (+/- sem), are combined from three experiments.

Figure 6.21

A Northern blot autoradiograph taken from one of the three experiments combined above. The lanes are aligned left to right with the treatments depicted left to right in Figure 6.20.


AT1 mRNA (% basal)
(normalised to β-actin mRNA)
100
80
60
40
20
0
*** p<0.005
***
Basal
Cortisol 10μM 6h
Cortisol 10μM 48h
1
2
3

maximal at this point. After 48h, AT_1 receptor mRNA levels had recovered to some extent but had not reached control levels.

The extent of the decrease induced by 10μM cortisol was then assessed quantitatively. Bovine zfr cells were treated with 10μM cortisol for 6 and 48h (Figures 6.20 and 6.21). Figure 6.20 represents a combination of the data from three experiments expressed as mean +/- s.e.m of AT_1 receptor mRNA levels as a percentage of basal, while Figure 6.21 shows a representative Northern blot autoradiograph from one of these experiments. The decrease induced by 10μM cortisol was significant ($p<0.005$) after 6 hours treatment, causing a fall in AT_1 receptor mRNA levels to 59% of control. Again a recovery in AT_1 receptor mRNA levels was observed for chronic treatment, with the level returning to 70% of control after 48 hours treatment.

6.2.7 : Effect of serum on the homologous regulation of AT_1 receptor mRNA levels

Normally, experiments which examine regulation of mRNA levels are carried out on serum-depleted cells. This makes the cells quiescent and allows gene regulation to be studied with no masking of the effects by the activated state of the cells. As the AT_1 receptor is involved in growth, however, it was decided to carry out experiments in serum-containing medium to determine what, if any, effect serum would have on the regulation of AT_1 receptor mRNA levels.

Bovine zfr cells were cultured as previously described but, instead of serum depriving all the flasks, some were maintained in serum during the course of the experiment. Certain flasks were treated with 10nM AII for four or eight hours. It can be clearly seen that serum increased the level of AT_1 receptor mRNA expression (Figure 6.22); however, this appeared to be a non-specific effect since the levels of β-actin mRNA were also increased. Also noticeable was the fact that in the absence of serum, 10nM AII induced

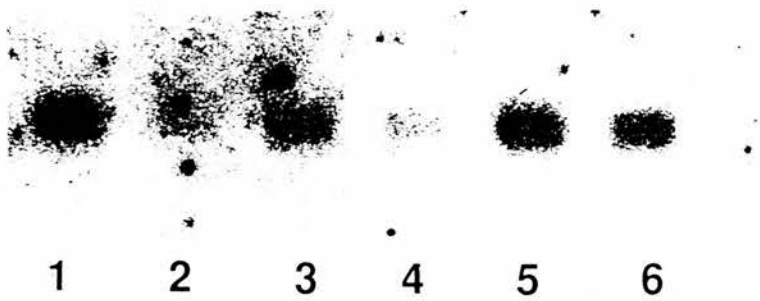


Figure 6.22

Effect of serum on levels of AT_1 receptor mRNA. Bovine zfr cells were cultured as described for two days. Half were then serum deprived and certain flasks were incubated with 10nM AII for 4 or 8 hours. Lanes 1, 3 and 5 are control samples, lanes 2,4 and 6 were incubated with 10nM AII for 4 hours (2) and 8 hours (4, 6) respectively. Lanes 1-4 were incubated in the absence of serum, whereas lanes 5 and 6 had serum present throughout the experiment

Treatment	Cortisol n-fold increase over basal
AII (10nM, 6h)	30.6
AII (10nM, 48h)	19
AII (1nM, 6h)	10.6
PMA (1μM, 6h)	16.3
PMA (1μM, 48h)	3.7
A23187 (1μM, 6h)	5
A23187 (1μM, 48h)	2.3
A+P (1μM, 6h)	3
A+P (1μM, 48h)	1.7
8Br-cAMP (0.1mM, 6h)	37
8Br-cAMP (0.1mM, 48h)	89.7
ACTH (1nM, 6h)	58.3
ACTH (1nM, 48h)	59.7
IGF-1 (10ng/mL, 6h)	6.3
IGF-1 (10ng/mL, 48h)	5.3
K+ (12mM, 6h)	7.3
K+ (12mM, 48h)	8.7

Table 6.3

Shown are changes in basal cortisol secretion observed in bovine zfr cells treated with the above agonists at the concentrations and times listed. Cortisol content was assayed in media removed from cells during the three quantitative experiments to study changes in AT_1 mRNA levels. All treatments increased cortisol secretion in the cells, even when the direction of regulation was different. Secretion was normalised to length of exposure for one flask of cells. For untreated flasks, basal cortisol secretion was 15 +/- 2pmol cortisol/5 x 10^6 cells/h.

a pronounced decrease in the mRNA levels of the AT_1 receptor, whereas in the presence of serum this decrease was not observed.

6.2.8 Steroidogenic response of the treated bovine zfr cells

Since an effect of cortisol on levels of AT_1 receptor mRNA had been seen in the previous experiments, it was wished to analyse the amount of cortisol present in the overlying media from the cells in the quantitative experiments. Media were collected at 24 and 48 hours of chronic incubations, and at the end of the 6 hour incubations, and assayed for cortisol content. The results from these assays are displayed in Figure 6.23; the combined data from three experiments are shown for the maximally effective concentration and time for each agonist used (except for cortisol).

Two points can be drawn from this data. Firstly, that, as expected in a zona fasciculata preparation, ACTH and 8-Br-cAMP were more effective at stimulating basal cortisol production than was AII. The IP_3/Ca^{2+} system agonists were also not as effective as AII itself. All these agents had elicited a decrease in AT_1 receptor mRNA levels; however, IGF-1 and K^+, which increased the receptor mRNA levels, also stimulated an increase in basal cortisol production.

6.3 Discussion

In this chapter, it was wished to examine the regulation of steady-state levels of AT_1 receptor mRNA in primary cultures of bovine adrenocortical cells. The results of these studies demonstrate that not only can angiotensin II regulate the levels of AT_1 receptor mRNA, but that activation of adrenocortical second messenger systems, and high concentrations of cortisol, can also modulate AT_1 receptor mRNA expression.

The decrease in AT_1 receptor mRNA induced by AII was first noticeable within 4 hours and thus precedes the decline in protein levels previously observed in bovine adrenocortical cells (Penhoat *et al.* 1988). Those studies, using ^{125}I-AII binding, showed a decrease in the number of binding sites with no loss of affinity after 24h treatment. Recent studies in H295R cells (Bird *et al.* 1995a) also found that AII caused a decrease in both AT_1 receptor mRNA and receptor protein (shown by radioligand binding); the changes in receptor mRNA levels preceded those in receptor protein by around 6h. Levels of both mRNA and protein recovered after chronic stimulation, in agreement with our results (Figure 6.2). In the light of these findings, we would expect AII receptor protein to have followed this pattern in our experiments.

AII, however, does not downregulate its own receptors in all tissues. High levels of AII upregulate adrenal AT_{1b} receptor mRNA in rats and also AT_1 receptor number and mRNA levels in cultured rat kidney tubule cells (Cheng *et al.* 1995). These, however, are probably species dependent differences. The AT_1 receptor genes have different regulatory regions in the two subtypes found in rats and mice, in contrast to the one class in humans and bovines. Regulation of AT_1 receptors in these latter two species can not, therefore, be accomplished through different regulatory sequences in two AT_1 subtype genes, but may be through the action of tissue-specific transcription factors.

We have further shown that regulation of the AT_1 receptor by its ligand, AII, can be mimicked by activating the IP_3/Ca^{2+} second messenger system. Stimulation of bovine zfr cells with either phorbol ester or calcium ionophore induced a decrease and recovery in AT_1 mRNA similar in magnitude and duration to that induced by AII. This indicates that both PKC (activated by phorbol ester) and calcium influx (stimulated by calcium ionophore) are involved in the decrease induced by AII. There is contrasting evidence as to whether the PKC or the calcium arm of the signal transduction system plays the major part in this effect. From our data, it would seem to be the PKC which predominates, as the decrease induced by PMA was more significant and mirrored the AII effect more closely than did the A23187. A similar effect was also demonstrated in H295R cells (Bird *et al.* 1995a). This group reported that PMA and calcium ionophore in combination could fully duplicate the decrease in AT_1 receptor mRNA levels induced by AII, but that phorbol ester alone could not. This observation is intriguing when considered with the fact that PKC has been reported to be less important in AII-stimulated actions than calcium (Ganguly & Waldron, 1994; Kapas *et al.* 1994). The data presented by Penhoat, examining AII receptor regulation by radioligand binding in bovine adrenocortical cells (Penhoat *et al.* 1988), showed on the other hand a greater effect of calcium ionophore in decreasing AII binding sites. However, both components (i.e. PMA and A23187) were necessary to fully duplicate the magnitude of the acute response of AII binding sites to AII. When considering the chronic response to AII observed in our studies, full activity of both arms of the IP_3/Ca^{2+} signal transduction system would not seem to be involved in the observed recovery in levels of AT_1 receptor mRNA : the PMA/A23187 combination prevented the recovery in AT_1 receptor mRNA levels observed during chronic AII treatment.

Calcium influx and PKC stimulation do not always exactly mimic AII's effects on its receptors, however. Following cloning of an AT_1 receptor (Makita *et al.* 1992), it was shown that inhibition of PKC did not affect the

AII-induced decrease of endogenous AT_1 receptor mRNA in cultured rat glomerular mesangial cells. A23187 did not mimic AII in this respect either, suggesting that the PKC/Ca^{2+} pathway was not involved in the regulation of AT_1 receptors by AII in these cells. This also suggests that although in adrenocortical cells regulation of AII receptors by AII may be mediated by activity of the IP_3/Ca^{2+} system, it is not the only mechanism by which it occurs.

Our findings indicate that activation of the cyclic AMP signal transduction system can also decrease levels of AT_1 receptor mRNA expression, but that this regulation does not mimic that induced by AII itself. In bovine adrenocortical cells, the cAMP system does not act so rapidly on AT_1 receptor mRNA levels as in the human, where an effect on AT_1 receptor mRNA levels was maximal within 3 hours of stimulation with dibutyryl cAMP (Bird *et al.* 1995a). In our studies the effect was not maximal until 48 hours after stimulation, but the degree of decrease was greater : 17% of control levels of AT_1 receptor mRNA after 48 hours incubation with 8-Br-cAMP, as opposed to 30% after 3h dibutyryl cAMP treatment in H295R cells. The different cAMP analogues appear to have acted at different speeds to decrease AT_1 receptor mRNA; it is possible that structural variation between the two analogues contributed to this discrepancy.

Whereas we observed a decrease to 17% of control levels of AT_1 receptor mRNA after 48h 8Br-cAMP treatment, Penhoat et al, who also used 8-Br-cAMP, observed a decrease to only 60% of control levels of AII receptor numbers in bovine adrenocortical (zfr) cells after 24 hours treatment (Penhoat *et al.* 1988). In our hands, ACTH was less effective than the cAMP analogue at decreasing AT_1 receptor mRNA levels, whereas Penhoat's group found the opposite (Penhoat *et al.* 1988). The reason for the greater efficacy of the second messenger analogue compared to the physiological ligand of the cAMP system, ACTH, is not clear. It may, however, be due to the more direct effect of 8-Br-cAMP mimicking greater levels of cAMP than the ACTH was able to transduce. It may also reflect differing effects of the two

compounds on AT_1 receptor mRNA levels and receptor protein. However, a greater efficacy of the cAMP analogue in decreasing AT_1 binding sites and receptor mRNA levels was also observed in H295R cells (Bird *et al.* 1995a).

The effect of the cAMP signal transduction system on AII receptors has been investigated in other tissues. Chen *et al.* (1994) demonstrated a transitory decrease in AT_1 mRNA expression in cultured rat kidney glomerular cells after treatment with forskolin, a direct activator of PKA, after which AT_1 mRNA levels increased to a level twice that of the control cells. Thus PKA would appear to induce a bi-directional regulation of the AII receptor. The mechanism for this effect is not clear.

Angiotensin II is known to exert its effects in some tissues through tyrosine kinase activation and opening of calcium channels (Cohen *et al.* 1988; Ganguly *et al.* 1995; Marrero *et al.* 1995). Might these signals also affect AT_1 mRNA levels? Our studies have shown that chronic exposure (48h) of zfr cells to IGF-1 caused an increase in AT_1 receptor mRNA levels, confirming the finding of Langlois et al, who demonstrated increases in AT_1 receptor mRNA expression in cultured bovine adrenocortical cells in response to a number of growth factors, including IGF-1 (Langlois *et al.* 1994). We additionally demonstrated an acute effect of this locally produced growth factor, which caused a decrease in levels of AT_1 receptor mRNA following 6h exposure. This acute response to IGF-1 treatment parallels the effect of AII, indicating that tyrosine kinases could be possibly be involved in AII-induced AT_1 receptor mRNA decreases in the adrenal cortex. However, the chronic responses differ, leading to the conclusion that long-term AII effects on its own receptor mRNA levels are probably not mediated through tyrosine kinases.

It is known that potassium can open voltage-dependent calcium channels in the human adrenal cortex (Laird *et al.* 1991). Our results from zfr cells treated with 12mM potassium, despite high variability, indicated a moderate but statistically insignificant increase in AT_1 mRNA levels. The increase was highest (124%) after 6 hours, then decreased to 114% after 48

hours, indicating that protracted high potassium has the reverse effect to protracted high AII. However, a higher concentration of potassium (16mM) tended to downregulate the receptor mRNA (84% after 48 hours), and a recent study in H295R cells also demonstrated that 14mM potassium significantly increased AT_1 mRNA levels and AT_1 receptor protein (Bird *et al.* 1995c).

From these data, two hypotheses can be advanced. The first is that potassium has a threshold effect on AT_1 receptor mRNA levels in adrenocortical cells. Up to a certain level, an increase will be observed, but beyond that the regulation is reversed. This hypothesis would go some way to explaining the high variation in our experiments with potassium : some preparations of zfr cells might be more sensitive to potassium than others. These cells would exhibit a decrease in AT_1 receptor mRNA levels in response to potassium at a lower concentration than would less sensitive cells. An increase in levels of AT_1 receptor mRNA would be observed in response to 12mM potassium in these less sensitive cells, but at the higher concentration a decrease of AT_1 receptor mRNA levels would be seen.

The alternative hypothesis relates to the mode of action of potassium in regulating AT_1 receptors. Potassium has been shown to open voltage-gated calcium channels in the adrenal cortex zona fasciculata (Laird *et al.* 1991; Bird *et al.* 1995c) and thus presumably modulates steroidogenesis, and AII receptors, through the actions of calcium-calmodulin protein kinases, similar to the calcium component of the IP_3/Ca^{2+} system in aldosterone synthesis. These kinases may well exhibit species differences, and this would account for the different regulatory responses to the same agonist observed in the two different species (I.M.Bird, personal communication). It could also account for the increase observed following high dietary potassium of AT_1 mRNA in rat adrenals (Lehoux *et al.* 1994) compared to the response to high potassium observed in human adrenal cells (Bird *et al.* 1995c). However, as it has previously been demonstrated that dietary potassium loading increases AII receptors in the rat adrenal but decreases them in rat vascular smooth

muscle (Douglas, 1979), this particular species difference may again reflect the subdivision of the AT_1 receptor class in rats and its unity in bovines and humans.

In vivo, plasma potassium concentrations of the magnitude used in these experiments would be incompatible with life, and the concentration needed to produce an increase in steroid secretion in freshly isolated cells is much lower (less than 8mM : Kojima *et al.* 1985). However, in these experiments, potassium was not detrimental to the cells and the concentrations used were those that had elicited maximal steroidogenic responses. It is therefore a distinct possibility that cell culture alters the cell's sensitivity to high potassium levels.

In the past, steroids have been discounted as regulators of angiotensin II receptors. Campanile and Goodfriend (1981) published a study in which many different steroids were tested for their ability to inhibit binding of AII to its receptor in the adrenal cortex and act as feedback modulators. None had any effect at physiological concentrations and steroids were discounted as being able to regulate adrenal AII receptors.

The finding in our study that cortisol increased AT_1 receptor mRNA levels in cultured bovine zfr cells is interesting in more than one way. Firstly, it suggests that a steroid short-loop feedback system is in operation in these cells, as Campanile & Goodfriend (1981) tried to show. Such a system could provide a mechanism for limiting cortisol production in response to raised AII concentrations. The adrenal cell could limit this by decreasing the amount of AII receptors capable of coupling to steroid production and therefore the amount of cortisol produced.

Another interesting facet of these results is the inability of aldosterone to regulate AT_1 receptor mRNA expression. This leads to postulation of the existence of zone-specific regulation by steroids. Thus, in the zona glomerulosa, aldosterone might downregulate AT_1 receptor mRNA expression, as cortisol does in the zona fasciculata. Cortisol would be

without effect in the zona glomerulosa, as these cells would not be exposed to high cortisol concentrations.

Other recent studies have indicated that in tissues other than the adrenal cortex, both mineralocorticoids and glucocorticoids can also act as regulators of AII receptors, particularly the AT_1 subtype. Most of this work has been done in vascular tissues to examine whether steroidal modulation of AT_1 receptors could be a potential mechanism for glucocorticoid-induced hypertension. Dexamethasone has indeed been found to upregulate AT_1 receptors (at both protein and mRNA levels) in cultured vascular smooth muscle cells (Gruenfeld & Eloy, 1987; Sato *et al.* 1994), as has aldosterone (Ullian *et al.* 1992). Therefore steroids are capable of modulating the AII receptor, and can do this bidirectionally, as there have been reports of glucocorticoids downregulating AT_1 receptor mRNA levels in hepatocytes (Wintersgill *et al.* 1995) as well as foetal ovine liver and kidney (Segar *et al.* 1995). This latter group also found that cortisol increased AT_1 receptor mRNA levels in heart and vascular smooth muscle, a further example of reciprocal regulation of AII receptors in different tissues.

Following the finding that cortisol, the product of AII and ACTH stimulation in bovine zfr cells, could downregulate AT_1 receptor mRNA expression, it was necessary to determine whether this was responsible for the effects seen in treatment with these agonists and their analogues. Finding such an indirect effect of cortisol (rather than a direct effect of the agonists used) would have diminished our previous results. The cortisol secreted into the overlying medium during experiments was therefore measured. The findings were quite conclusive. The amount of cortisol secreted into the medium bore no absolute relation to the degree or direction of the regulation of AT_1 receptor mRNA levels observed. PMA/A23187 in combination, for instance, decreased the levels of AT_1 receptor mRNA to the same extent as AII for 6 hours but produced 6 times less cortisol. ACTH and 8-Br-cAMP did not downregulate the AT_1 receptor mRNA levels to the same extent as AII, but the cells treated with these compounds secreted 2 and 3 times more

cortisol. K^+ and IGF-1, which both increased AT_1 receptor mRNA levels, increased basal secretion of cortisol to the same level as the PMA/A23187 combination, which almost completely depleted the cells of AT_1 receptor mRNA. Therefore, although high levels of cortisol do downregulate AT_1 receptor mRNA levels in bovine zfr cells, this is not the only mechanism by which the decrease is effected.

The last variable to be examined in this series of experiments was the effect of serum on the expression and regulation of AT_1 receptor mRNA. Serum increases many genes, as it promotes the cell to an actively growing state. This is particularly pertinent to the expression of angiotensin receptors, as AII is itself a growth factor. It is not surprising, then that the presence of serum increased AT_1 receptor mRNA, but it is more surprising that this increase was persistent. 10nM AII in normal experimental conditions decreases AT_1 receptor mRNA expression to 16% of control within 6h. However, when the same experiment was conducted in the presence of serum, this regulation was not observed. An explanation for this could be drawn from the results obtained with IGF-1, and with bFGF (Langlois *et al.* 1994). The growth factors present in serum could be upregulating the receptor to such an extent that this masks the decrease induced by AII, and thus no apparent change is seen.

The findings from the experiments presented in this chapter are subject to certain limitations. Firstly, a degree of variability has been observed in the results from some of these experiments, particularly with regard to incubations of bovine zfr cells with IGF-1 and potassium. In large part, this is due to the experimental model system i.e. primary cell cultures. Because these are by their nature obtained from different sources for each preparation, there will be variations in the responsiveness and other characteristics of each preparation. Therefore, although the trend observed may be the same, the magnitude of the effect may vary. This can lead to variation when results are combined from different experiments. Slight differences in experimental technique between experiments will also play a

part, as may inter-preparation variations in agonist sensitivity. Minimising such variation is one of the advantages of using a tumour-derived cell line, such as the human adrenocortical cell line H295R. This cell line, derived from an invasive primary adrenocortical carcinoma, has become available as a mixed adrenocortical cell line producing over 20 steroids and is much used as a model for human adrenal cells (Gazdar *et al.* 1990).

There are many advantages to these cells. The main among them is that this is a human cell line and thus the problem of species difference can be minimised. Also the problem of variance in primary cultures discussed above is also minimised. Longer term experiments can also be attempted as the cells do not lose their enzymes and die as in primary cultures (see also Chapter 3 discussion); the H295R cells divide and are stable indefinitely.

However, there are also disadvantages. Zone-specific studies can not be attempted in these cells as they are a multipotent cell line, that is they produce steroids from all three zones of the cortex. They can be influenced to one pathway, e.g. mineralocorticoid, but not exclusively. The H295R cells also have different proportions of steroidogenic enzymes than in primary cultures of human adrenocortical cells : the level of aldosterone synthase is much lower and consequently much less aldosterone is synthesised; they also have much lower levels of ACTH receptors than primary cultures. This can obviously influence the results of experiments involving these variables. Lastly, as H295R cells are derived from an adrenal tumour, they are by definition less differentiated than an early primary culture; this may also influence experimental results.

Secondly, in this study, experiments were carried out to examine changes in mRNA steady-state levels only. We can therefore only speculate as to how the decrease in AT_1 receptor mRNA level is achieved. The early time points at which decreases in AT_1 receptor mRNA levels were observed would seem to indicate that mRNA synthesis is being switched off in some way, or possibly that it is being destabilised. The former possibility could be assessed by performing RNA run-on/run-off assays, which would yield

information about the transcription of the AT_1 receptor mRNA. The consequent recovery in mRNA following long-term treatment with AII is harder to explain; it is possible that the loss of synthesis or destabilisation is only short-lived, or that in response to this, the AT_1 receptor DNA is transcribed at a higher rate; this may then lead to the observed increase.

Another possible limitation of these experiments was that cortisol, which can act as a mineralocorticoid in some situations, was used instead of a 'pure' glucocorticoid like dexamethasone. Criticism of this can be answered by the fact that aldosterone, a mineralocorticoid, was absolutely without effect on AT_1 mRNA levels in these experiments. It is therefore extremely unlikely that cortisol was acting as a mineralocorticoid in this context.

The results presented in this chapter have broadened our knowledge of the regulation of AT_1 receptors in this important model tissue. Levels of AT_1 receptor mRNA can be regulated both homologously and heterologously, with activation of multiple signal transduction systems known to operate in the adrenal cortex affecting basal levels. Cross-talk between signal transduction systems could therefore have complex effects on both AII receptors and AII action. The changes in levels of AT_1 receptor mRNA appear to precede observed changes in AII receptor protein, suggesting that the major level of AII receptor regulation is its transcription. Evidence has also been presented for the existence of a steroid short-loop feedback system in the adrenal cortex, indicating that the AT_1 receptor can be modulated by as well as its agonist.

↳ the steroid produced as a result of its stimulation

Chapter 7 : General Discussion

The aims of this thesis were to study the nature and regulation of angiotensin II receptors in the bovine adrenal cortex. To this end, existing protocols for the culture of bovine adrenocortical zona glomerulosa cells were modified. A simpler method for purification of the cells following collagenase dispersion was introduced, and it was demonstrated that the addition of antioxidants was not necessary for maintenance of steroid secretion. These antioxidants, recommended by many investigators (Crivello *et al.* 1982; Braley *et al.* 1992; Shepherd *et al.* 1992), were in fact found to have an inhibitory effect on steroid secretion for the first two days in culture. Experiments to elucidate the particular antioxidant responsible for this effect, or what in our culture medium was responsible for maintaining responsiveness to AII, ACTH and potassium, would be a fascinating extension to these studies. A fuller characterisation of the purified culture (e.g. cytospin analysis of cell types following purification, analysis of changes in AII receptor number and affinity during culture) would also complement the results presented here.

To resolve an interesting observation that AT_1 receptors in cultured bovine zfr cells might be pharmacologically distinct from those in rabbit vascular smooth muscle (Clyne *et al.* 1993), Schild analysis was performed in two cell culture systems : bovine adrenal zg cells and rat mesenteric artery vascular smooth muscle cells. This, while revealing that the AT_1 receptors in the two zones of the adrenal cortex were pharmacologically identical and not significantly different from those in rat vascular smooth muscle cells, pointed at another interesting observation. The pA_2 values generated for cultured rat vascular smooth muscle cells were significantly different from those obtained from organ bath studies on rabbit aortic smooth muscle strips. The possibility is therefore raised that Schild analysis to determine pA_2 values may not be as widely comparable a technique as was thought. One way to resolve the observed discrepancy would be to perform a similar

Schild analysis to that performed in this thesis on rat mesenteric artery vascular smooth muscle, but using the isolated perfused mesenteric artery. This would then be a more comparable method to that which generated the pA_2 value for losartan in the rabbit aorta (Chiu *et al.* 1990). If a difference in pA_2 values were then obtained, this would raise a very important consideration that pA_2 values might not be as comparable between species as was originally thought (Mackay, 1978).

Having demonstrated that the AT_1 receptors in the inner and outer zones of the bovine adrenal cortex were pharmacologically indistinguishable, it was possible to focus on the bovine zfr culture as a model for regulation of the AII receptor. The coding sequence of the AT_1 receptor generated from PCR of bovine genomic DNA was shown through sequence analysis to be at least 98% identical with the published sequence for this receptor (Sasaki *et al.* 1991). However, certain discrepancies remained after the sequence analysis had been performed. Two areas of each clone analysed (pAT_1.2C and 44) could not be adequately sequenced along both strands due to CG-rich regions inducing compressions of the sequence data. The present data, however, does not preclude that the PCR-generated sequences are identical to the published sequence. To complete this analysis, therefore, more analysis using either primers designed against different bases of the coding region to those used previously, or other available dGTP analogues, should be performed; the latter may prove to be the better starting point.

The last part of the work in this thesis has examined the regulation of AT_1 receptor mRNA steady-state levels in cultures of bovine adrenocortical inner zone cells. The data produced has shown that this regulation is multifactorial : AT_1 receptor mRNA levels can be affected by not just AII but also second messengers active in the adrenal cortex. This raises interesting questions about the mechanisms by which the regulation is brought about, which is the controlling influence, and what the physiological significance of the changes observed might be.

Simultaneous activation of the signal transduction systems studied in this thesis may have contrasting or synergistic effects on the levels of AT_1 receptor mRNA, and as all the systems studied are active in the adrenal gland, dissection of which are the major influences may be difficult. Cross-talk between second messenger systems clearly has important implications for the expression of AT_1 receptors. As a second messenger rarely works alone during cell stimulation, correlation of AT_1 receptor mRNA regulation to a single intracellular signal in an adrenocortical cell culture system is not feasible.

From the results in presented in this thesis, it would seem that the regulation by AII of its receptor mRNA levels is principally mediated by the IP_3/Ca^{2+} signal transduction system. However, as both arms of the system appear to mimic the effects of AII, it may be difficult to determine the exact mechanism by which the effect is mediated.

Regulation of AII receptors in bovine adrenocortical cells by AII and ACTH has been demonstrated to be similar in both zfr and zg cells, in that both secretagogues decrease the number of binding sites without affecting affinity (Penhoat *et al.* 1988). AII and ACTH have also been shown to decrease AT_1 receptor mRNA levels in cultured human adrenocortical cells (Naville *et al.* 1993). Both AII and ACTH are stimuli for aldosterone secretion, so it is not surprising that they should both regulate zg AII receptors in the same direction as zfr AII receptors. Indeed, a discrepancy could affect the maintenance of normal aldosterone secretion. The significance of ACTH regulating AII receptors in the zfr is less clear. It may perhaps reflect the fact that although AII will cause the zfr to secrete cortisol, it will not do this in the presence of fluctuating ACTH levels. This was suggested by the findings of Mason *et al.* (1979) who demonstrated secretion of cortisol from human adrenals *in vivo,* in response to AII, only in the presence of an ACTH clamp. AII-stimulated cortisol secretion is therefore secondary to ACTH-stimulated cortisol secretion : maybe the decrease of AII receptors by ACTH ensures that this has to be so.

The mechanisms behind the regulation of AT_1 receptor mRNA levels by ACTH and cAMP are not fully understood. According to Makita *et al.* (1992), there are no cAMP-responsive elements in the AT_1 receptor gene promoter region, therefore they postulated that the effects were post-transcriptional in nature, involving changes in mRNA stability. The same group reported that cAMP-induced decrease of rat AT_1 receptor mRNA was not dependent on protein synthesis, whereas AII-induced decrease was, thus providing further evidence for the differing mechanisms of regulation by the two systems, as suggested by the different timescales observed in bovine zfr cells.

The mechanism of the decrease in AT_1 receptor mRNA levels induced by cortisol is also not certain. The AT_1 receptor in rodents is known to have a glucocorticoid response element (Guo *et al.* 1995); given the similarities in genomic organisation and receptor regulation in different species, it is likely that a similar element is also present in bovines and that this is immediately responsible for the regulation observed.

It is interesting to speculate on how the regulatory agents investigated in these studies might affect the expression of AT_1 receptor mRNA *in vivo*. The adrenal gland is exposed to AII at both a local and a systemic level. Plasma AII may be more likely to modulate acute effects of AII on its own receptor, while the local renin-angiotensin system could be involved in long-term regulation in the adrenal cortex. Raised concentrations of IGF-1 and K^+ are probably locally generated. An intriguing picture emerges that influences which act to downregulate the AT_1 receptor mRNA levels are systemically generated, while those acting to raise the levels are locally generated (here the action of AII in raising its receptor mRNA levels on chronic exposure can be included).

The physiological implications of the observed masking of regulation of AT_1 receptor mRNA by serum are less clear. The significance would depend partly on whether this masking of regulation was present for all the downregulators examined, or solely for AII, and partly on whether this

'growth factor' was present in normal serum or plasma. If it were purely a serum and not a plasma factor, there should not be much effect on AT_1 receptor regulation *in vivo*. However, if the factor were present in plasma, it could well reach the AT_1 receptors in adrenocortical cells and influence their regulation to an unknown degree. If, for some reason, the amount of postulated 'growth factor' were to be abnormally high, this could have significant effects on AT_1 receptor expression and steroidogenic responses to angiotensin II.

The results presented in this chapter have broadened our knowledge of the regulation of AT_1 receptors in this important model tissue. However, further work is needed to expand this knowledge to yield a fuller picture of the nature, significance and mechanisms of AII receptor regulation. First to be addressed would be the performance of ligand binding experiments to examine how closely the receptor protein follows the mRNA in response to the agonists studied. Use of transcription inhibitors, protein synthesis inhibitors and run-on/run-off assays would also be needed to assess at what level the observed regulation is taking place and whether it varies between agonists. The experiments should also be extended to the zona glomerulosa, if the low cell yield can be overcome (use of RT-PCR to assess AT_1 receptor mRNA levels is a possibility) as the possible existence of zone-specific regulation should not be ignored. This could add another dimension to the theories of adrenocortical zonation.

Co-ordinate regulation of AT_1 and AT_2 receptors has been proposed, shown in some tissues (Kijima *et al.* 1995), and refuted in others (De Gasparo *et al.* 1994). It would therefore be most interesting to examine the changes in AT_2 receptor mRNA levels following incubation of both zg and zfr cells with the agonists used previously. Under normal conditions virtually no AT_2 receptors are present, and therefore any increase would be very exciting.

The challenge of dissecting out which signal transduction systems plays the major part in regulating the AT_1 receptor could be initiated by expressing the AT_1 receptor in non-steroidogenic cells (e.g. CHO cells) in

which only one system is known to be active. Other systems could then be mimicked and alterations in regulation of the expressed receptor determined. Another attractive possibility would be to study some of the agonists used in a bovine vascular smooth muscle cell culture system. Examination of how steroids might affect this non-steroidogenic tissue should prove interesting.

Lastly, once the mechanism by which the receptor is regulated has been determined, isolation of any putative tissue-specific transcription factors responsible for inducing this regulation would be fascinating and also may provide a way to manipulate AT_1 receptor regulation should this be possible or necessary.

In concluding this chapter, I would like to consider the potential pathophysiological significance of the results obtained. The renin-angiotensin system is highly important in regulating blood pressure and volume. The AT_1 receptor is a significant part of this system, and if its regulation were disturbed, so then might be the responses to AII in its target organs. This can be envisaged by considering the effect of ACTH on AT_1 receptor regulation. Chronically raised ACTH, as well as increasing cortisol, may downregulate AT_1 receptors, and thus decrease adrenal sensitivity to AII. This could in turn lead to disturbances in aldosterone secretion and electrolyte balance. To fully understand the implications of disturbance of AII receptor regulation, it is essential to understand both the nature of AII receptors and how their regulation is effected. The results presented in this thesis show that both are highly complex; the regulation especially appears to involve the interplay of many intracellular systems, which may act at a local or a systemic level.

Appendix 1 : Publications

The following publications have arisen from the work presented in this thesis :

GC Dell, SD Morley, JJ Mullins, BC Williams and SW Walker (1996): Regulation of Angiotensin II (AT_1) receptor expression in bovine adrenocortical cells. *Journal of Endocrinology* **148** : OC8

GC Dell, SD Morley, JJ Mullins, BC Williams and SW Walker : Multiple signal transduction systems regulate type 1 Angiotensin II receptor mRNA expression in bovine adrenocortical cells. *Endocrine Research* **22** : 363-368

GC Dell, SD Morley, JJ Mullins, SW Walker and BC Williams : Regulation of type 1 angiotensin II receptor (AT_1) mRNA expression in bovine adrenocortical cells by multiple signal transduction systems and adrenocortical steroids. *Endocrinology;* in preparation.

ENDOCRINE RESEARCH, 22(4), 363-368 (1996)

MULTIPLE SIGNAL TRANSDUCTION SYSTEMS REGULATE ANGIOTENSIN II TYPE 1 (AT_1) RECEPTOR mRNA EXPRESSION IN BOVINE ADRENOCORTICAL CELLS

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ABSTRACT

Regulation of AT_1 receptor mRNA expression is an important determinant of angiotensin II-induced steroidogenesis. We have PCR-amplified the bovine adrenal AT_1 receptor coding region using primers designed from the published bovine AT_1 receptor sequence. This has been used as a probe on Northern blots to detect changes in the levels of AT_1 receptor mRNA in primary cultures of bovine zona fasciculata cells in response to activation of several different signal transduction mechanisms in additon to two major adrenal steroid products, cortisol and aldosterone. AT_1 receptor mRNA decreased in response to 6hr AII (10 nM) treatment, but returned to basal levels following 48h AII treatment. This effect was mimicked by the phorbol ester PMA (1 μM) and the calcium ionophore A23187 (1 μM), both singly and in combination. Activation of the cAMP pathway by ACTH (1 nM) and 8-bromo-cAMP (0.1 μM) also decreased AT_1 receptor mRNA levels. In contrast, both IGF-1 (10 ng/ml) and potassium ions (12 mM) increased the levels of AT_1 receptor mRNA. Finally, cortisol (10 μM) but not aldosterone (100nM) decreased AT_1 receptor mRNA. We conclude that the regulation of AT_1 receptor mRNA in bovine zona fasciculata cells could involve several different signal transduction systems in addition to adrenocortical steroids themselves.

INTRODUCTION

Angiotensin II (AII) acts through specific AT_1 receptors to regulate aldosterone secretion from the zona glomerulosa of the adrenal cortex in most mammalian species including the human. In the human and bovine species AII also stimulates cortisol secretion from the zona fasciculata (zf) (1, 2). Bovine adrenocortical cells provide a useful model for the human since they exhibit similar steroidogenic responsiveness (3). AII receptor activation in bovine zf cells is coupled to the inositol trisphosphate/calcium (IP_3/Ca^{2+}) second messenger system (4). Previous studies in bovine zf cells demonstrated that prolonged exposure to AII or to effectors of both cyclic AMP (cAMP) dependent kinases and protein kinase C decreased the binding of ^{125}I-AII, consistent with a loss of AII receptors (5). A similar pattern of down-regulation of AT_1 receptors was inferred by analysis of AT_1 receptor mRNA levels in the H295R human adrenocortical tumour cell line (6).

In this study, we report on the regulation of AT_1 receptor mRNA in primary cultures of bovine zf cells. It has now become clear that the control of cortisol secretion in zf cells is multifactorial and since multiple signal transduction mechanisms are present in adrenocortical cells, we chose to study the effects of AII itself, the phorbol ester PMA, the calcium ionophore A23187, ACTH, (which acts through cAMP), 8-bromo-cAMP, insulin-like growth factor-1 (IGF-1, a stimulator of tyrosine kinase) and potassium, which activates voltage-gated Ca^{2+} channels (7,8). In addition we tested whether the two principal steroid products of the adrenal cortex, namely cortisol and aldosterone, had the potential to directly regulate AT_1 receptor mRNA.

METHODS

The bovine adrenal AT_1 receptor coding region was amplified by PCR with primers designed from the published AT_1 receptor sequence (9), using genomic DNA as the template, and inserted into a modification of pSP72 (Promega).

Bovine zf cells were isolated and cultured as described previously (10). On the second day after isolation, the cells were serum deprived for 6 h before being exposed to agonists for the stated times and concentrations. Medium containing agonists was changed daily. At the end of the treatment period, total RNA was isolated from the cells using RNAzol reagent (11). 25μg RNA was analyzed by Northern blotting (12) and hybridized to a ^{32}P-labelled probe for the AT_1 receptor, prepared by random priming (13) of the cloned AT_1 receptor coding region. Experiments were performed in duplicate.

RESULTS

Exposure of bovine zf cells to 10 nM AII resulted in a rapid and dramatic decrease in AT_1 receptor mRNA, which was detectable after 4 h and maintained until 24 h, but returned to basal levels after 48 h. A similar time-course of decrease, followed by recovery of AT_1 receptor mRNA was observed after exposure of the cells to either 1 μM-phorbol ester (PMA) or calcium ionophore (A23187). However, when these two agonists were applied in combination, AT_1 receptor mRNA showed the same rapid decrease, but remained depressed after 48 h (Table I). Treatment of the cells with either 1 nM ACTH, or with 0.1 mM-8-bromo cAMP, also caused a decrease in AT_1 receptor mRNA (Table I). This decrease was first apparent after 8 h, and reached its maximum after 48 h. Acute exposure (8 h) of cells to 10 ng/mL IGF-1 produced a decrease in AT_1 receptor mRNA, which recovered and indeed was increased above basal levels after 48 h exposure (Table I). Potassium (12 mM) also increased AT_1 receptor mRNA, reaching a maximal effect after 48 h treatment (Table I). Aldosterone (100 nM) had no effect on AT_1 receptor mRNA. In contrast, cortisol (10 μM) induced a pronounced decrease in AT_1 receptor mRNA in these cells (Table I).

DISCUSSION

These results, although qualitative in nature, indicate the complexity of AT_1 receptor mRNA regulation in bovine zf cells. AII induced a rapid decrease in

TABLE I

Effect of acute and chronic treatment with various agonists on AT_1 receptor mRNA in bovine zf cells (↓ decrease; ↑ increase; ↔ unchanged).

Agonist	AT_1 receptor mRNA level (acute)	AT_1 receptor mRNA level (chronic)
AII (10 nM)	↓↓	↔
PMA (1 μM)	↓↓	↔
A23187 (1 μM)	↓	↔
PMA + A23187 (1 μM)	↓↓	↓↓
ACTH (1 nM)	↓	↓↓
8-Bromo-cAMP (0.1mM)	↓	↓↓
IGF-1 (10 ng/mL)	↓	↑↑
Potassium (12 mM)	↑↑	↑
Cortisol (10 μM)	↓↓	↓
Aldosterone (100 nM)	↔	↔

AT_1 receptor mRNA. This down-regulation could be mimicked by exposure to PMA or A23187 singly or in combination. However, after chronic incubation with the PMA/A23187 combination, levels of AT_1 receptor mRNA did not recover as they did after AII treatment. These results compare with published data on AII receptor binding in bovine zf cells (5) except that the decrease in AT_1 receptor mRNA in our study occurred more acutely after exposure to these agonists than did the decrease in AT_1 receptor binding previously reported (5). In H295R cells, Bird et al. also observed that changes in AII receptor binding lagged behind changes in AT_1 receptor mRNA by some 6 h in response to AII and PMA/A23187 (6). Although both AT_1 receptor mRNA and AII receptor binding levels appear to respond similarly to cAMP activators and analogs in both bovine and human species, these changes nevertheless occur on different time scales. In the human H295R cells, maximal decrease in AT_1 mRNA occurred after 6 h stimulation (6),

whereas in bovine zf cells we observed a greater decrease after 48h both with ACTH and 8-bromo-cAMP. In contrast to the effects of cAMP, the tyrosine kinase agonist, IGF-1, caused an acute decrease followed by a more chronic and pronounced increase in AT_1 receptor mRNA. This finding is in keeping with the results of other workers (14). Potassium is the only agonist which led to both an acute and chronic increase in AT_1 receptor mRNA in bovine zf cells and this contrasts with recent results from H295R cells, which showed that 14 mM potassium produced a significant decrease in AT_1 receptor mRNA (6). The reason for this discrepancy is not clear and requires further investigation. Adrenal steroids have not previously been considered to be important in the physiological regulation of AII receptors (15). However, we observed that cortisol, but not aldosterone, produced both an acute and chronic decrease in AT_1 receptor mRNA. These findings suggest that cortisol can directly regulate AT_1 receptor expression at the level of transcription. Clearly further experiments are required in order to elucidate this novel finding.

In conclusion we have demonstrated that AT_1 receptor mRNA can be differentially regulated by several separate signal transduction mechanisms and potentially by adrenocortical steroids themselves.

ACKNOWLEDGMENTS

The award of a Medical Research Council Studentship to G.C.Dell is gratefully acknowledged.

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